

**CHRONIC ULTRAVIOLET IRRADIATION:
EFFECTS ON IMMUNE RESPONSES AND
TUMOUR OUTGROWTH**

**Thesis submitted for the Degree of Doctor of Philosophy by
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Abstract

Exposure to solar ultraviolet (UV) radiation causes induction of skin cancers. Previous experiments using mice have demonstrated that the majority of these skin tumours are antigenic, and are rejected upon transplantation into immunocompetent, syngeneic recipients. However, prior irradiation of the recipients with sub-carcinogenic doses of UV renders them susceptible to progressive tumour outgrowth, suggesting a role for UV in suppression of immune responses to skin tumours. Using a moderately immunogenic fibrosarcoma (FSA) cell line, the present study examines which UV wavebands are most effective in increasing susceptibility to tumour cell outgrowth; it also investigates the mechanisms by which UV exposure affects immune responses to injected tumour cells.

To establish the most important wavebands for affecting the immunity to tumours, three different lamps were used: a broad-band UVB source (TL12), a narrow-band UVB source (TL01, 311-313 nm) and a UVA-I source (340-400 nm). A three week irradiation protocol was chosen as this has previously been shown to result in increased outgrowth of implanted tumours. The immunosuppressive properties of these lamps were assessed by irradiating mice twice a week for three weeks. All three sources were shown to reduce the number of epidermal Langerhans cells and the contact hypersensitivity response. Additionally, the numbers of dendritic cells in the draining lymph nodes of mice irradiated over 3 weeks with the broad-band UVB source were counted; a significant reduction in lymph node dendritic cell numbers was observed in irradiated mice compared with unirradiated

controls, although the expression of MHC Class II and CD86 antigens on these cells was unaffected.

The effects of the different lamps on tumour outgrowth were assessed by irradiating mice twice a week for three weeks on their shaved backs; the FSA cells were then injected subcutaneously into the irradiated site. Prior exposure of mice to doses of 1200 J/m², but not 1000 J/m² broad-band UVB resulted in increased growth of the injected cells, indicating a dose dependency of UVB-enhanced tumour outgrowth. No increase in tumour size was seen following injection of FSA cells into an unirradiated site on UV-irradiated mice, demonstrating that the effect of the UVB was a local one. Prior exposure of mice to narrow-band UVB or UVA-I did not result in increased tumour cell outgrowth.

In unirradiated tumour-bearing mice, it was found that the *in vitro* spontaneous proliferation of cells from lymph nodes draining the sites of tumour cell injection was increased compared with the proliferation of cells from tumour-free mice. Taking lymph nodes at various time points after tumour cell inoculation showed that UVB-exposure prior to FSA cell injection resulted in a delay in the time at which an increase in the *in vitro* proliferation became apparent. Using flow cytometry, the expression of various cell surface markers on lymph node cells from tumour-bearing mice was assessed. Additionally, an attempt was made to identify any infiltrating host cells in the tumours using immunohistochemistry.

Cis-urocanic acid (UCA), formed from the naturally occurring *trans*-isomer on UVB-irradiation of skin, is a recognised initiator of UVB-induced immunosuppression, and has previously been implicated in photocarcinogenesis. In the present study, mice were treated twice a week for 3 weeks with *cis*-UCA prior to

FSA cell injection; this treatment did not result in increased outgrowth of the resulting tumours. Similarly, injection of a monoclonal antibody with specificity for *cis*-UCA prior to each irradiation with broad-band UVB did not reverse the UV-induced increase in tumour growth. Thus no role for *cis*-UCA in UV-enhanced tumour outgrowth was established.

List of Abbreviations

APC	Antigen presenting cell
ATPase	Adenosine triphosphatase
BCC	Basal cell carcinoma
CFC	Chlorofluorocarbon
CH	Contact hypersensitivity
Con A	Concanavalin A
cpm	counts per minute
DC	Dendritic cell
DETC	Dendritic epidermal T cell
DLN	Draining lymph node
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
EC	Epidermal cells
FITC	Fluorescein isothiocyanate
FSA	Fibrosarcoma
$\gamma\delta$	gamma-delta
HSV	Herpes simplex virus
ICAM-1	Intercellular adhesion molecule-1
IFN- γ	Interferon-gamma
IL	Interleukin
LC	Langerhans cell
MED	Minimum erythematous dose
MHC	Major histocompatibility complex
mRNA	messenger ribonucleic acid
NGF	Nerve growth factor
NK	natural killer
NMSC	Non-melanoma skin cancer
PAF	Platelet-activating factor
PHA	Phytohemagglutinin
SCC	Squamous cell carcinoma
TA	Tumour antigen

TCR	T cell receptor
TGF- β	Transforming growth factor-beta
Th	T-helper
TNF- α	Tumour necrosis factor-alpha
UCA	Urocanic acid
UVR	Ultraviolet radiation

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Chapter 1

General Introduction

1.1 - Ultraviolet Radiation

1.1.1 - Environmental exposure

The ultraviolet (UV) spectrum is divided into UVC (200-280 nm), UVB (280-315 nm) and UVA (315-400 nm). The UVA waveband is further sub-divided into UVA-II (315-340 nm) and UVA-I (340-400 nm). The major source of UV exposure of humans is sunlight. Of the UV that reaches the earth's surface the majority is UVA, with some UVB. All of the wavelengths in the UVC range, and some UVB is absorbed by ozone, which forms a thin layer in the stratosphere. In the early 1980s a hole in the ozone layer was discovered above the Antarctic, which is present each year from spring to autumn. This, along with thinning of the ozone layer globally, raised concerns that increasing amounts of UVB, which are the most biologically active wavelengths, would reach the earth's surface. A long-term study in Poland found that between 1976 to 1997, there was an increase in UV intensity, capable of causing sunburn at the terrestrial surface, of approximately 6% per decade. This was attributed mainly to ozone change (Borkowski, 2000). In the Antarctic, the increase was 130% (Madronich *et al*, 1998).

Ozone depletion is primarily due to release of chlorofluorocarbons (CFCs) and the Montreal Protocol of 1987 limits the use and manufacture of CFCs. Although it is likely that it will be some years before the full benefits of these measures will be seen, modelling studies have demonstrated that ozone column amounts in the Southern hemisphere are 5% more than they would otherwise have been; it is expected that it will

be 50 years until the ozone layer is repaired, with a probable delay due to global warming (McKenzie *et al*, 2003).

1.1.2 - Artificial sources

Other sources of human UV exposure include UV lamps used in the treatment of medical conditions such as psoriasis, and lamps used for cosmetic tanning.

Additionally, there may be some accidental exposure to industrial UV sources, such as welding arcs.

1.1.3 - Biological effects

One benefit of UV exposure is the production of vitamin D₃ by photoisomerisation of 7-dihydrocholesterol. Studies have also found an association of UV exposure with reduced incidence of some internal cancers, such as prostate cancer (Luscombe *et al*, 2001). Damaging effects of UV exposure include sunburn, photoageing of the skin, immune suppression, ocular effects and skin cancer. Increasing UV exposure of humans is due to a number of factors, including longer life expectancy and changes in lifestyles and attitudes, such as increased leisure time, changes in clothing and the desire for tanned skin. In many countries, the rising popularity of sunshine holidays is the most important factor in causing increased UV exposure; estimates of the UV dose received during a 3 week summer holiday in the USA demonstrated that this can increase the annual UV dose of an average American by around 30% (Godar *et al*, 2001). This increase in sun exposure is mirrored by an increased incidence of skin cancer, as discussed in the next section.

1.2 - Skin Cancer

1.2.1 - Incidence worldwide

Non-melanoma skin cancers (NMSC) are the most common malignant neoplasms in humans. These include squamous cell carcinoma (SCC, Figure 1.1), a malignant neoplasm derived from keratinocytes of the squamous epithelium, and basal cell carcinoma (BCC, Figure 1.2), derived from the cells of the basal lamina. Numerous studies have shown that cases of NMSC in Caucasian populations continue to increase. A study in south Wales found that from 1988-1998 the number of total cases of NMSC rose from 173.4 to 265.4 per 100,000 population (Holme *et al*, 2000).



Figure 1.1. Squamous cell carcinoma

In Australia, the current figures for NMSC are extremely high; a study in Queensland between 1997-1999 found that the yearly age-standardised incidence of BCC per 100,000 population was 1444.8 for men and 942.7 for women. For SCC the rates were

805 for men and 423.6 for women (Raasch and Buettner, 2002). Other studies in Sweden and the USA have demonstrated that the incidence of NMSC has greatly increased over the last 30 years (Dahl *et al*, 1992, Karagas *et al*, 1999).



Figure 1.2. Basal cell carcinoma

Melanoma (Figure 1.3), although less prevalent than the NMSC, is the most common fatal malignancy in young adults; many studies have shown that the incidence of melanoma has also increased, for example in Norway (Moan and Dahlback, 1992) and the USA (Elder, 1995). A Scottish study (MacKie *et al*, 2002) found that from 1979 to 1998 melanoma incidence per 100,000 increased from 3.5 to 10.6 for men, and from 7.0 to 13.1 for women.

1.2.2 - Sunlight exposure and skin cancer

The risk factors for skin cancer point to a role for sunlight exposure. The incidence of NMSC is higher in areas of high ambient solar irradiance, such as Australia, related to occupational exposure to sunlight, and increased on sun-exposed parts of the body (Kricker *et al*, 1994). Skin cancer is more prevalent in Caucasian populations, compared with those with more pigmented skin. In a Californian study, the incidence of melanoma in Blacks was 1.0 (female) and 0.7 (male) per 100,000 population, whereas the incidence in non-Hispanic whites was 17.2 (female) and 11.3 (male) (Cress and Holly, 1997).

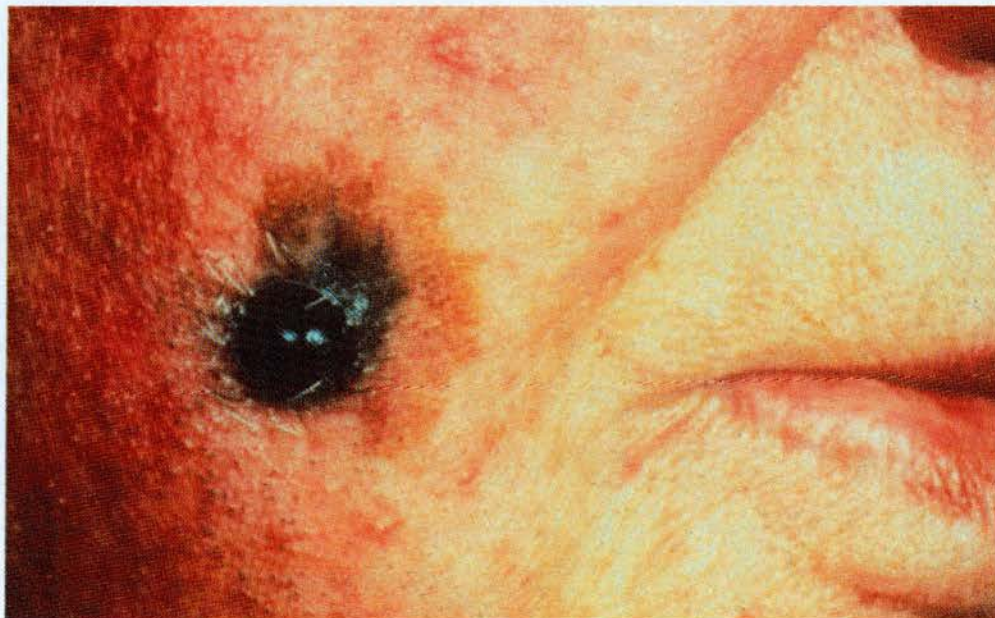


Figure 1.3. Melanoma

The most important risk factor for SCC is thought to be cumulative lifetime sunlight exposure (Vitasa *et al*, 1990; English *et al*, 1998). SCC is found mostly on the

most sun exposed sites, such as the upper face, neck, ears and hands, and can progress by stages; sun-damaged epithelium, actinic keratosis, carcinoma *in situ* and SCC.

The role of sunlight exposure in BCC is less clear. Sun exposure appears to induce BCC at lower levels; this is thought to be due to a lower dose of radiation being required for transformation of the highly mitotic cells of the basal lamina, compared with the keratinocytes that give rise to SCC (Rosso *et al*, 1996). One study found that the incidence of BCC in Norway increased with decreasing latitude (Moan and Dahlback, 1992); however, this has not been seen in other studies (Kricker *et al*, 1994). BCC are commonly found on sun exposed sites, including the head and neck. However, BCC is increasingly found on sites, such as the trunk, that are exposed less frequently to the sun (Kricker *et al*, 1994). A study in western Australia suggested that intermittent exposure to sunlight caused increased risk of BCC compared with a more continual exposure (Kricker *et al*, 1995).

Like BCC, the incidence of cutaneous malignant melanoma is not linked to cumulative lifetime sunlight exposure; melanoma is more common in indoor, rather than outdoor workers, and as well as being found on the face and neck, it is found on less frequently exposed areas such as the trunk (males) and lower limbs (females) (Elder, 1995). Studies in humans have suggested that melanoma may arise as a result of high dose, intermittent sun exposure, particularly during childhood (Holman *et al*, 1983; Whiteman *et al*, 2001). Two animal studies have substantiated this observation. Using transgenic mice that have melanocytes in a similar distribution to human skin, Noonan *et al* (2001) demonstrated that a single dose of UVR (23 minimal erythral doses, MED) of neonatal (3.5 days) but not adult (6 weeks) mice was sufficient to induce melanoma later in life. In a model using the opossum *Monodelphis domestica*, 3 weeks

exposure of sucklings to moderate doses of UVB resulted in the development of cutaneous melanoma (Robinson *et al*, 1998).

1.2.3 - UV and induction of skin cancer

In a study of photocarcinogenesis in hairless mice, it was demonstrated that the wavelength of maximum effectiveness for tumour induction was 293 nm (in the UVB range); there was also a shoulder of maximum effectiveness above 340 nm (UVA) (de Gruijl *et al*, 1993). UVB induces characteristic lesions in cellular DNA, in the form of pyrimidine dimers. Mutations arise during DNA replication, when DNA polymerase incorporates an adenine residue (A) opposite the pyrimidine photoproducts; the resulting C→T (and less commonly CC→TT) transitions are signature mutations of UVB-induced DNA damage, and are detectable in human NMSC (Brash *et al*, 1991; Ziegler *et al*, 1993). UVA wavelengths are thought to indirectly damage DNA through the formation of reactive oxygen species, which cause oxidative damage, including single-strand breaks (Kielbassa *et al*, 1997). Mutations in genes that code for regulators of the cell cycle, including tumour suppressor genes, and oncogenes, have been shown to be important in the pathogenesis of skin cancer.

DNA damage by UV induces expression of the tumour suppressor gene p53, leading either to transient cell cycle arrest (Fisher, 1994) or apoptotic cell death (Clarke *et al*, 1993), which helps to prevent survival of cells containing DNA damage. Mutations in the p53 gene are thought to be an early event in the pathogenesis of skin cancer (Berg *et al*, 1996; Ziegler *et al*, 1994), and occur in over 90 % of human SCC, with 58 % being C→T or CC→TT (Brash *et al*, 1991); p53 mutations are also seen in human BCC (Rady *et al*, 1992). p53 mutations are also present in 20-100 % tumours in

UV-irradiated mice, the majority having C→T or CC→TT mutations (Kress *et al*, 1992; Kanjilal *et al*, 1993). UV radiation also induces expression of another tumour suppressor gene, p16^{INK4}, which binds to cyclin-dependent kinases to block cell cycle progression (Pavey *et al*, 1999). Inactivated p16 is seen in the majority of melanoma cell lines (Rizos *et al*, 1999); and UV-induced C→T mutations in the p16 gene have been seen in melanoma *in vivo* (Herbst *et al*, 1997).

In mice, the proto-oncogene *ras* appears to be essential for melanoma development (Chin *et al*, 1999); around 70 % of human melanomas display aberrant activation of the gene that codes for BRAF, a protein which is activated by *ras*, although they do not show a signature UVB mutation (Davies *et al*, 2002). UV-induced SCC of mice display increased *ras* gene expression (Husain *et al*, 1990). However, the role of *ras* mutations in development of human NMSC is less clear (Campbell *et al*, 1993).

1.2.4 Sunscreens and skin cancer

Until recently, there was little evidence that sunscreen use prevents skin cancer. Two short-term studies had shown that sunscreen application over 7 (Thompson *et al*, 1993) or 24 (Naylor *et al*, 1995) months reduced the appearance of solar keratoses. Protection from skin cancer by sunscreen use was shown in a recent study by Green *et al* (1999), in which adults in Queensland, Australia applied sunscreen daily on the head, neck, arms and hands for a period of 4.5 years. Compared to the non-sunscreen group (who applied sunscreen at their own discretion) the incidence of SCC in those that applied sunscreen daily was significantly reduced from 1832 to 1115 per 100,000. Sunscreen application had no significant effect on BCC however, possibly because SCC

induction is related to long-term sunlight exposure, whereas BCC is thought to be related to intermittent sun exposure. Evidence that sunscreen protects against melanoma and BCC is lacking; however, when considering the possible link between early incidences of sunburn and melanoma, and intermittent sun exposure and BCC, it might be speculated that use of sunscreen earlier in life, reducing incidences of sunburn, may protect against the induction of these skin cancers.

1.3 - Cutaneous immunity

1.3.1 - Skin physiology

The skin is the largest organ of the body and is exposed to a wide variety of exogenous influences including UV radiation, microorganisms, chemicals and physical damage. It is relatively impermeable to water and has an important barrier function. The structural integrity of the skin is provided mostly by the dermis, which consists of connective tissue of collagen and elastin fibres, into which capillaries and lymphatics intertwine. Outside the dermis is the epidermis, made up of keratinocytes predominantly, the outermost layer of which is the stratum corneum, comprising dead cells which then slough off.

The skin is the first point of defence against many microorganisms, and provides both a physical barrier and protection through inhibitory substances, such as antimicrobial peptides. In addition, the skin has a number of cells that can lead to specific, protective immune responses (Figure 1.4); these cells make up the skin immune system (SIS; Bos and Kapsenberg, 1986). Leukocytes in the blood extravasate through the blood vessel wall to populate the tissue of the skin. Cells migrating out of

the skin do so via the afferent lymphatics, which carry leukocytes from the tissues to the draining lymph nodes (DLN).

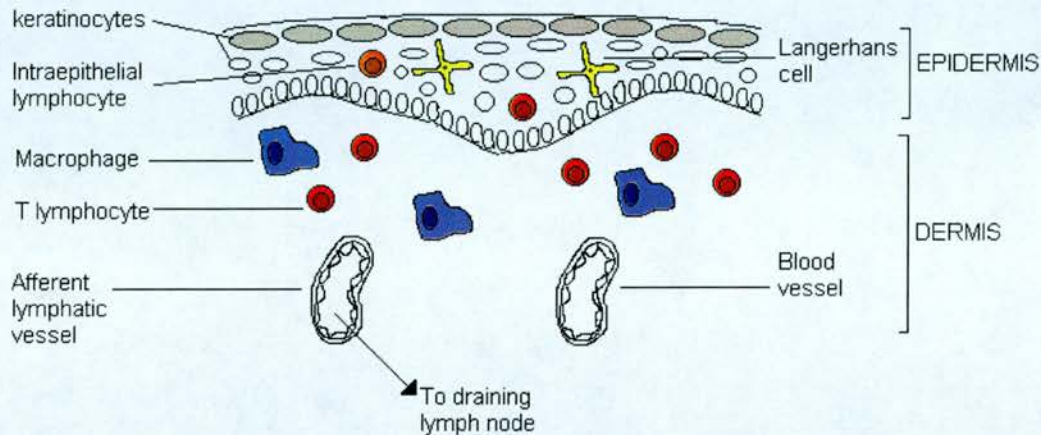


Figure 1.4. Cellular components of the skin immune system.

1.3.2 - Keratinocytes

The majority of skin cells are keratinocytes, which make up the growing component of the epidermis. In addition to this role, keratinocytes can express/release numerous molecules involved in induction of immune responses. Human and murine keratinocytes express the major histocompatibility complex (MHC) class II surface antigen, involved in presentation of antigen to T cells (Section 1.3.6), upon exposure to IFN- γ (Wilkner *et al*, 1986). IFN- γ is produced by activated T cells and natural killer (NK) cells, and its main actions include activation of macrophages, upregulation of MHC class I and II expression, and promotion of the differentiation of CD4⁺ T cells towards the Th1 subset (section 1.3.6). IFN- γ exposure also upregulates keratinocyte

expression of intercellular adhesion molecule (ICAM) -1 (Dustin *et al*, 1985), a ligand for T cell binding.

Keratinocytes release a number of cytokines, such as the inflammatory cytokines tumour necrosis factor (TNF)- α and interleukin (IL) -1 (Kutsch *et al*, 1993; Kock *et al*, 1990). The main actions of TNF- α include induction of adhesion molecule expression on vascular endothelial cells and activation of macrophages; IL-1 has similar inflammatory actions to TNF- α . Murine keratinocytes have also been demonstrated to produce the anti-inflammatory IL-10 (Enk *et al*, 1992), although studies have failed to observe IL-10 production by human keratinocytes (Teunissen *et al*, 1997; Jackson *et al*, 1996). IL-10 is also produced by Th2 T cells, and inhibits the effector functions of Th1 cells; IL-10 acts to reduce production of pro-inflammatory cytokines such as IFN γ and TNF- α , and decrease expression of co-stimulatory molecules on antigen presenting cells (APC).

The expression of surface receptors, including MHC class II, that are important in T cell recognition of antigen would suggest that keratinocytes could act as APC to induce T cell responses; however, there is no definitive evidence that keratinocytes can initiate immune responses in this way.

1.3.3 - Langerhans cells

Langerhans cells (LC) are members of the bone-marrow derived dendritic cell lineage, and constitute around 1 % of the cells of the epidermis. They have long dendritic processes that extend through the epidermis to form a meshwork. LC are professional antigen presenting cells (APC) and express high levels of MHC class II; other surface molecules expressed on LC, that are used in combination for detection of

these cells, include CD1a (human LC), and the enzyme adenosine triphosphatase (ATPase). LC also contain unique intracellular rod-shaped organelles known as Birbeck granules (Birbeck *et al*, 1961), the function of which is unclear.

LC are potent APC, capable of internalising antigen, processing it and presenting it to T cells. Following application of antigen to the skin, LC phagocytose the antigen and migrate to the afferent lymphatics, where they are known as veiled cells. They are carried in the lymph to the DLN, and differentiate into interdigitating cells, expressing high levels of MHC Class II, enabling efficient antigen presentation to T cells in the paracortex of the DLN. For T cell activation, both antigen presented by MHC class II molecules and a second, co-stimulatory signal is required. LC can be induced to express molecules involved in interaction with T cells, such as ICAM-1 (Teunissen *et al*, 1994), and B7.1 and B7.2, which bind to CD28 on T cells to provide a co-stimulatory signal. LC also release T cell activating cytokines, including IL-1 and IL-12. The principal sources of IL-12 are macrophages and dendritic cells; IL-12 causes T cells to produce IFN- γ , promotes differentiation of Th1-type CD4⁺ cells, and enhances the cytotoxic activities of CD8⁺ T cells and NK cells.

1.3.4 - Dermal APC

Some of the DC found in the dermis are LC precursors that are migrating from the blood vessels towards the epidermis, or are LC moving from the epidermis to the afferent lymphatics. There is also a distinct subset of DC resident in the dermis, that lack CD1a, BG, and ATPase; the majority of these cells appear to be more related to macrophages, expressing CD68 and CD11b (Narvaez *et al*, 1995). In addition, cells expressing macrophage markers such as CD14 are present (Nestle *et al*, 1993). The

antigen presenting ability of dermal cells has been shown by Tse and Cooper (1990), who demonstrated that dermal cells taken from mice and exposed to antigen *in vitro* are capable of induction of an immune response upon injection into naïve syngeneic mice.

1.3.5 - Mast cells

Mast cells contain numerous secretory granules, and degranulation results in release of various preformed mediators, including histamine and TNF- α . Activation of mast cells can occur following exposure to a number of stimuli, such as IgE and neuropeptides. Release of histamine causes smooth muscle contraction and increased vascular permeability, resulting in an influx of cells and fluid into the tissues. Dermal mast cells have been implicated in UV-induced immunosuppression, as will be discussed in section 1.5.5 below.

1.3.6 - T cells

T lymphocytes express a unique T cell receptor (TCR) which is antigen-specific, and which requires antigen presentation in conjunction with MHC molecules. There are two main subsets of T cells, CD4⁺ and CD8⁺. CD8⁺ T cells recognise antigen presented by MHC class I molecules, which are present on all nucleated cells, and which generally present antigens that have originated from within the cell, such as viruses, or tumour cell antigens. Following antigen recognition and activation of specific CD8⁺ cells, these cells then lyse the affected cell. CD4⁺ T cells (T helper [Th] cells) require antigen presentation by professional APC that process and present antigen via the MHC class II pathway; antigens presented in this way are mostly exogenous, that have been phagocytosed by the APC. Activation of CD4⁺ T cells results in release of cytokines,

and CD4⁺ T cells have been further sub-divided into Th1 and Th2 cells, based on the repertoire of cytokines that they release. For example, Th1 cells release IFN γ , which stimulates phagocytes, and IL-2, which is an autocrine T cell growth factor, and stimulates CD8⁺ T cells. Characteristic cytokines produced by Th2 cells include IL-4, IL-5 and IL-10; as well as being involved in some protective immune responses such as antibody production, some Th2 cytokines can suppress the production of Th1 cytokines.

The majority of skin associated T cells are found in the dermis, with a few detectable in the epidermis. Most of these are memory T cells, that have previously been exposed to antigen, and many of these express the homing receptor cutaneous lymphocyte-associated antigen (CLA), that binds to E-selectin, which is expressed on blood vessels within the skin (Berg *et al*, 1991). Both CD8⁺ and CD4⁺ T cells are represented in the skin. Humans and mice differ in that the majority of T cells in human skin express the $\alpha\beta$ form of the TCR, while in murine skin, a significant population of dendritic epidermal $\gamma\delta$ -TCR T cells is present.

1.3.6 - Hypersensitivity responses

Contact hypersensitivity (CH) responses are induced following application of a chemical to the skin, and are characterised by redness and swelling in the sensitised site. Extensive experimental evidence indicates that during the sensitisation stage of CH, epidermal LC internalise the antigen and migrate to the draining lymph nodes (DLN), where they stimulate differentiation and proliferation of naïve T cells (Kimber *et al*, 1990; Kripke *et al*, 1990); these T cells then migrate back to the site of skin sensitisation. Elicitation of CH occurs following challenge of previously sensitised animals, with a peak response at around 24 h after exposure; migration of the already

expanded T cells into the challenge site results in a much increased hypersensitivity response and leukocyte recruitment than that seen after a single application. Th1 cells are thought to be the most important cells in mediating CH, promoting increased release of proinflammatory cytokines, including IL-1, IFN- γ and TNF- α (reviewed in Ullrich, 1995).

Delayed type hypersensitivity reactions are mounted following subcutaneous exposure to more complex antigens such as allogeneic cells, or infection with a microorganism.

1.4 - Immunity to cutaneous tumours

1.4.1 - Skin cancer and immunosurveillance

The theory of immunosurveillance states that the immune system protects against the development of cancers, by recognition and elimination of transformed cells (Burnet, 1970). Control of tumour outgrowth by the immune system is thought to be particularly important in skin cancers. Histological examination of skin cancers in humans reveals a mononuclear inflammatory infiltrate around almost all SCC (Kohchiyama *et al*, 1986) and over 60% of BCC (Habets *et al*, 1988), with a few cells infiltrating the tumour nests. Immunohistochemical analysis of human BCC found that the majority of infiltrating cells were T cells (50-75%); the remaining cells included macrophages (16%), LC (4-15%) and NK cells (4%) (Habets *et al*, 1988; Kohchiyama *et al*, 1986; Synkowski *et al*, 1985). Additionally, a proportion of melanomas and BCC undergo spontaneous regression, accompanied by increased numbers of infiltrating T cells compared with non-regressing lesions (Hunt *et al*, 1994; Tefany *et al*, 1991).

Actinic keratoses, the pre-malignant stage of SCC, can also undergo spontaneous regression, which is thought to be due to a host immune response (Marks *et al*, 1986).

The incidence of skin cancer in patients who are being treated with immunosuppressive agents has been found to be greater than that in the general population. Numerous studies have found increased incidences of NMSC in renal allograft patients (Boyle *et al*, 1984; Hartevelt *et al*, 1990); in Australia the incidence was found to exceed 40%, 9 years post-transplant (Hardie *et al*, 1980). Studies of heart transplant patients have found that 15% of patients developed at least one cutaneous neoplasm (BCC or SCC), within 5 years of transplantation (Espana *et al*, 1995; Caforio *et al*, 2001). The incidence of melanoma in renal transplant patients is also increased (Greene *et al*, 1981). Patients receiving immunosuppressive agents for reasons other than transplantation also show higher incidences of pre-malignant and malignant skin lesions (Marshall, 1974). Anecdotal evidence also suggests that NMSC in immunosuppressed patients behave more aggressively (Weimar *et al*, 1979), implying that the immune system, as well as preventing the appearance of neoplastic lesions, also controls the outgrowth of existing tumours.

The immune system also appears to be important in experimentally UV-induced skin cancer in mice; a study of photocarcinogenesis in hairless mice demonstrated that treatment with the immunosuppressive drug azothioprine resulted in enhanced tumour induction (Koranda *et al*, 1975).

1.4.2 - Antigenicity of UV-induced tumours

Many skin tumours induced experimentally in mice by chronic UV exposure are antigenic, such that if these tumours are transplanted into normal mice, they are

rejected; however, the tumours grow progressively if they are transplanted into thymectomised, x-irradiated mice (Kripke, 1974). Mice immunised with tumour fragments were resistant to subsequent challenge with the same tumour, whereas mice immunised with a different tumour from that used for challenge were equally susceptible to challenge as unimmunised mice (Kripke, 1974). Thus, tumours induced by UV exposure express tumour-specific antigens (TSA), which are not found on other UV-induced tumours, or on normal cells. *In vitro* UV irradiation of a weakly antigenic melanoma cell line generated antigenic variants; an *in vivo* immunisation and challenge assay demonstrated that the immunity generated against one variant failed to protect against challenge with other antigenic variants (Hostetler *et al*, 1989). CD8⁺ cytotoxic clones generated by immunisation with UV-induced tumours, and maintained in culture, were found to be uniquely specific for the particular tumour used for immunisation, as measured by their *in vitro* cytotoxicity (Ward *et al*, 1989).

UV-induced tumours also express tumour-associated antigens (TAA); these are proteins that may be found on normal cells, but which are over-expressed on tumour cells. In humans, a number of melanoma antigens have been described, the majority of which appear to be either developmental antigens, or differentiation antigens of melanocytes (Pardoll, 1994).

1.4.3 - Antigen presenting cells

LC, by nature of their professional antigen presentation ability, and location in the epidermis, are the most likely cells involved in induction of immune responses to UV-induced skin tumours. The number of tumour infiltrating LC does not appear to indicate the effectiveness of the anti-tumour immune response (Smolle *et al*, 1986). An

increase in the number of LC in the epidermis overlying BCC has been observed (Yamaji *et al*, 1987); however, a decrease in epidermal LC in BCC has also been reported (Bergfelt *et al*, 1992). The morphology of LC near BCC is altered, showing thicker and fewer dendrites (Bergfelt *et al*, 1994). Presentation of tumour antigens by APC in humans was investigated in a study by Nestle *et al* (1998), where DC were generated from peripheral blood of melanoma patients and then pulsed with tumour antigen. Vaccination of the patients with these DC resulted in regression or partial regression in 5 out of 16 patients.

The majority of evidence for the role of antigen presentation in tumour immunity has come from animal models. Grabbe *et al* (1991) immunised mice 3 times at weekly intervals with Thy-1 depleted epidermal cells (EC) pulsed with tumour fragments of a chemically induced fibrosarcoma. Mice receiving tumour antigen-pulsed syngeneic EC demonstrated protective immunity to subsequent challenge with live tumour cells, and were also able to mount a DTH response to killed tumour cells; induction of immunity required culture of the EC in granulocyte-macrophage colony stimulating factor (GM-CSF) prior to antigen pulsing. Further studies demonstrated that this ability of epidermal LC to induce tumour immunity could be abrogated by culture of the cells in IFN- γ or IL-10, but not TNF- α (Grabbe *et al*, 1994; Beissert *et al*, 1995; Grabbe *et al*, 1992). Using this model, the ability of antigen-pulsed LC to elicit a DTH response in mice that had been immunised with whole tumour cells was also shown; this was inhibited by culture of the LC in IFN- γ or IL-10, but augmented by culture of the cells in TNF- α . The ability of APC to elicit a response to tumour antigen *in vitro* was demonstrated by Cohen *et al* (1994), where T cells from hyperimmune mice proliferated in response to tumour antigen-pulsed epidermal LC or splenic DC from naïve mice.

Dermal I-A⁺ cells pulsed with tumour-antigen in a similar system to that used by Grabbe *et al* (1991) were also able to induce tumour immunity when used to immunise mice; as seen with epidermal cells, these dermal APC could also elicit DTH responses in mice immunised with killed tumour cells (Campton *et al*, 2000).

Sluyter *et al* (2001) transplanted skin overlying subcutaneous tumours (resulting from injection of cells from a UV-induced fibrosarcoma) onto naïve mice. This resulted in induction of tumour immunity in the naïve mice, suggesting that APC in the epidermis and dermis of the tumour-bearing mice contained sufficient levels of antigen to induce an immune response in the recipient mice. Lucas and Halliday (1999) found that FITC-induced migration of LC from the epidermis overlying progressor tumours to the draining lymph nodes (DLN) was inhibited by a factor released by the tumours. LC migration was not inhibited from epidermis overlying regressor tumours however, implying that induction of an effective immune response to tumours requires migration of LC to DLN.

The $\gamma\delta^+$ DETC population resident in murine epidermis may also be important in tumour immunity. $\gamma\delta^{+/-}$ transgenic mice show increased susceptibility to an injected SCC line (Girardi *et al*, 2001). When mixed with murine melanoma cells, DETC have been shown to prevent tumour growth after intradermal injection (Love-Schimenti and Kripke, 1994). Following culture in IL-2, DETC cell lines show *in vitro* cytotoxicity towards a range of skin tumour cell lines, including melanomas (Kaminski *et al*, 1993) and UV-induced fibrosarcomas (Okamoto *et al*, 1988) but not normal keratinocytes. This effect is thought to be mediated by a surface glycoprotein, 2B4, which is also found on natural killer (NK) cells (Schuhmachers *et al*, 1995). DETC have also been implicated in induction of protective T cell immunity. Cavanagh *et al* (1997)

demonstrated that a single immunisation with tumour antigen-pulsed DETC, followed by a single immunisation with a low dose of live tumour cells induced protective, long-term tumour immunity.

1.4.4 - T cells

Studies of human BCC have found that the majority of tumour infiltrating T cells express markers of activated T cells (Synkowski *et al*, 1985; Habets *et al*, 1988).

Almost all of the T cells infiltrating human SCC were found to bear the $\alpha\beta$ TCR and these were demonstrated to be cytotoxic towards autologous tumour cells *in vitro* (Haeffner *et al*, 1997). Analysis of the variable β chain usage of infiltrating T cells from human BCC and SCC demonstrated that certain variable β chain families were overrepresented in tumour-infiltrating lymphocytes, compared with T cells from the blood of the same patient, suggesting that clonally expanded T cells may contribute to a specific immune response to NMSC (Ohmen *et al*, 1994).

Both $CD4^+$ and $CD8^+$ T cells are thought to be involved in the specific immune response to skin tumours. *In vitro* experiments have demonstrated that lysis of SCC cells by cell lines derived from autologous tumour-infiltrating lymphocytes is dependent on MHC class I expression (Chikamatsu *et al*, 1994), indicating that the cytotoxic immune response requires $CD8^+$ T cells. Expansion *in vitro* of cells from LN draining melanomas in mice resulted in a population of predominantly $CD8^+$ T cells, which showed specific cytolytic activity in a MHC class I restricted manner; adoptive transfer of these cells, with co-injection of IL-2, showed a significant anti-tumour effect against metastatic melanoma in mice (Harada *et al*, 1996).

Studies have found that, in regressing human BCC and melanomas, the number of infiltrating CD4⁺ cells was increased, whereas there was no difference in the number of infiltrating CD8⁺ and macrophages compared with progressing tumours (Hunt *et al*, 1994; Tefany *et al*, 1991), suggesting that CD4⁺ cells are most important in mediating skin tumour destruction.

Animal studies have also demonstrated the central role of CD4⁺ cells in immune responses to skin tumours. Regression of a UV-induced tumour cell line in mice was associated with an increase in helper T cell activity, which was detectable in the environment of the regressing tumour (Romerdahl and Kripke, 1988). Hung *et al* (1998) immunised wild-type, CD8^{-/-} and CD4^{-/-} mice with B16 melanoma cells, then assessed induction of protective tumour immunity by challenge with the melanoma cells. The majority of wild-type mice were able to mount a protective immune response; however, whereas none of the CD4^{-/-} mice were protected from tumour challenge; a significant proportion of the CD8^{-/-} mice were able to mount tumour rejection. It has also been demonstrated that transfer of CD4⁺ tumour-specific T cells into SCID mice can cause rejection of UV-induced tumour cells, and that this effect is dependent on IFN- γ ; additionally, the IFN- γ did not act directly on the tumour cells themselves, suggesting that tumour immunity by CD4⁺ T cells may be mediated by activation of effector cells such as macrophages (Mumberg *et al*, 1999).

1.4.5 - Other effector cells

NK cells are thought to play a role in tumour immunosurveillance by recognition and destruction of cells with decreased MHC class I expression, such as transformed cells. A decrease in MHC class I expression in BCC has been reported (Hua *et al*,

1985). Kohchiyama *et al* (1986) observed numerous NK cells in close contact with tumour cells in human SCC. Human BCC could be successfully transplanted into athymic mice with impaired NK cell function (nude mice), without the need for the further immunosuppression that is required in mice with intact NK cells (Grimwood *et al*, 1988). Additionally, transfer of human NK cells into NK cell-depleted SCID mice provided some protection against two human melanoma cell lines when co-injected with IL-2 (Hill *et al*, 1994).

Macrophages are also present in substantial numbers in the tumour infiltrate of human and murine skin tumours (Habets *et al*, 1988; McBride *et al*, 1992). They may contribute to cytotoxic killing of tumour cells, following activation by cytokines expressed by T cells; alternatively they may be involved in presentation of tumour antigens.

1.4.6 - Alteration of immune responses by tumours

A number of studies have found that tumours can cause suppression of immune responses, which may aid their escape from immunosurveillance mechanisms. Tumour infiltrating lymphocytes from melanoma and BCC patients demonstrated suppressed proliferative responses following *in vitro* mitogenic stimulation (Miescher *et al*, 1986). In the same study, it was seen that normal peripheral blood lymphocytes exposed *in vitro* to tumour cells or their supernatants also lost their ability to respond to mitogenic stimulation.

Measurement of cytokine levels following culture of whole blood from skin cancer patients found no alterations in cytokine production by cells from BCC patients; however, melanoma patients demonstrated decreased levels of IL-2, IFN- γ and TNF- α

(Elasser-Beile *et al*, 1993). It is possible that this may be a result of the tumours, or it may be that alterations in cytokines in these patients has allowed the outgrowth of the melanomas. Melanoma cell lines have been shown to produce certain cytokines in culture, such as IL-10 and TGF- β , which could have immunosuppressive activities (Bennicelli and Guerry, 1993).

Tumour cells may also interfere with antigen presentation; incubation of monocyte-derived DC in the culture supernatant from a melanoma cell line resulted in altered maturation of these cells, such that they were unable to produce IL-12; these cells then rapidly underwent apoptosis (Kiertscher *et al*, 2000).

1.5 - UV-induced immunosuppression

1.5.1 - Suppression of immune responses

A large number of human and animal studies have demonstrated the suppressive effects of UV exposure on immune responses, particularly cell-mediated immune responses. UV irradiation suppresses the induction of hypersensitivity responses in humans and mice (Yoshikawa *et al*, 1990; Noonan *et al*, 1981). Animal studies have shown that UVB exposure enhances susceptibility to infectious diseases, as determined by microbial load or mortality; decreased resistance to bacteria, fungi, parasites and viruses has been observed in irradiated animals. As will be discussed in section 1.6, UVB-induced immunosuppression also plays a role in outgrowth of skin tumours.

1.5.2 - Initiators of immunosuppression

UV exposure can cause local suppression of responses, when antigen is applied to the irradiated skin, or systemic suppression of responses to an antigen applied to a site

distant from the irradiated skin. Wavelengths within the UVB range do not penetrate past the upper layer of the dermis; therefore it is thought that to initiate systemic suppression, UV acts via a photoreceptor in the skin. Two skin chromophores that absorb UVB, and that have been shown to have a role in UV-induced immune modulation, are urocanic acid (UCA) and DNA, both of which are discussed below.

The *trans* isomer of UCA is formed by the deamination of histidine by the enzyme histidase (Figure 1.5). During keratinisation, the histidine-rich proteins of keratohyalin granules break down, and, once the cells become impermeable, the histidase enzyme becomes activated, converting histidine to UCA (Scott *et al*, 1982). The enzymes required for the metabolism of UCA are present in the liver, however they are absent from the skin, resulting in the accumulation of *trans*-UCA in the stratum corneum (Scott, 1981), such that it represents up to 0.7% of the dry weight of the epidermis.

The first evidence for UCA as a photoreceptor for UV-induced immunosuppression came from experiments by De Fabo and Noonan (1983), who demonstrated that the action spectrum of UV-induced suppression of CH matched the absorption spectrum of UCA, and that tape-stripping of murine skin to remove the stratum corneum prior to irradiation reversed UV-induced systemic suppression of CH.

On exposure of *trans*-UCA to UV, it is photoisomerised to form *cis*-UCA (Figure 1.5), in a dose-dependent fashion, until a photostationary state is reached, when the two isomers are present in approximately equal quantities. The most effective wavelengths for *cis*- to *trans*-UCA isomerisation in human skin were shown to lie between 280-310 nm (Gibbs *et al*, 1997), while the action spectrum for isomerisation in mouse skin has been shown to have a peak between 300-310 nm (Gibbs *et al*, 1993).

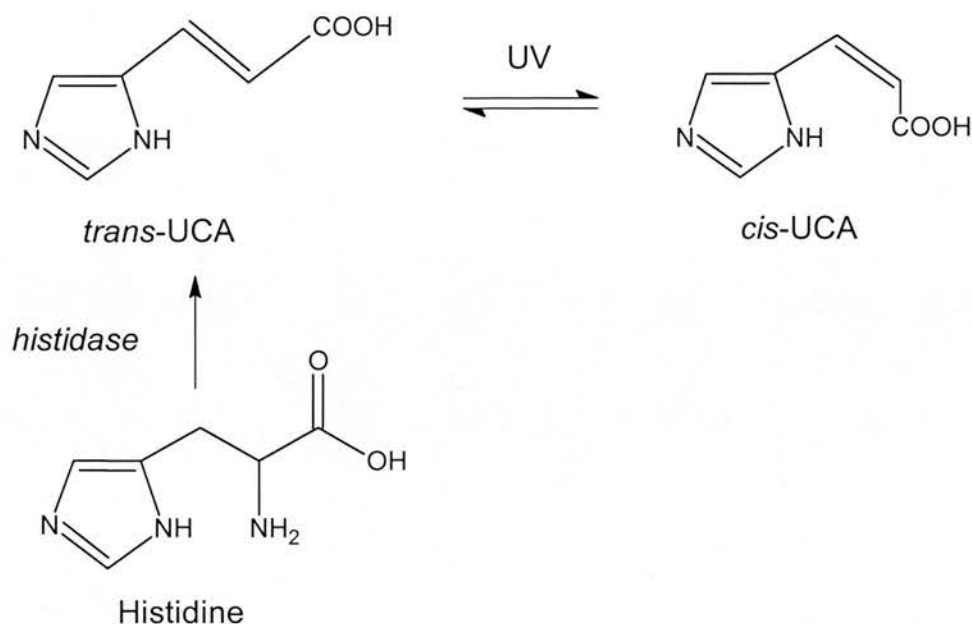


Figure 1.5. Biochemical formation and photoisomerisation of UCA isomers

Once formed *cis*-UCA persists in the skin for up to two weeks, and is shed along with dead keratinocytes and sweat. *Cis*-UCA has also been detected in the serum for at least two days after UVB-irradiation of mice (Moodycliffe *et al*, 1993) and in the urine of UVB-exposed human volunteers for up to 12 days after exposure (Kammeyer *et al*, 1997); thus, after UV exposure, *cis*-UCA is present systemically, and may act at sites distant from the skin.

A large number of studies in animal models have demonstrated the ability of *cis*-UCA to suppress immune responses. For example, treatment of mice topically with *cis*-UCA prior to contact sensitisation suppressed the CH response to oxazalone (Lauerma *et al*, 1995). Topical *cis*-UCA treatment also suppressed DTH responses to herpes simplex virus (HSV, Ross *et al*, 1986). Multiple subcutaneous injections of *cis*-UCA before and after oral infection of rats with the parasitic worm *Trichinella spiralis*

resulted in increased numbers of *T. spiralis* larvae in the muscle tissue of infected rats, and a significantly impaired DTH response to *T. spiralis* antigen (Garssen *et al*, 1999). Additionally, the development of a monoclonal antibody specific for *cis*-UCA (Moodycliffe *et al*, 1993) has permitted investigation of the role of *cis*-UCA in UV-induced immunosuppression. Injection of *cis*-UCA antibody prior to UVB exposure abrogated the UV-induced suppression of DTH to alloantigen (Moodycliffe *et al*, 1996), and UV-induced suppression of immune responses to *T. spiralis* in rats (Garssen *et al*, 1999). Injection of *cis*-UCA antibody prior to UV exposure did not reverse UV-induced suppression of CH however (El-Ghorr and Norval, 1995; Moodycliffe *et al*, 1996), suggesting that *cis*-UCA mediates some, but not all of the immunosuppressive effects of UV.

Cis-UCA may cause immune suppression via effects on antigen presenting cells. Application of *cis*-UCA to the skin caused a significant decrease in the number of epidermal LC (Norval *et al*, 1990), while injection of *cis*-UCA antibody prior to UV exposure prevented the UV-induced reduction in epidermal LC numbers (El-Ghorr and Norval, 1995). Dendritic cells taken from *cis*-UCA treated mice demonstrated significantly impaired APC ability *in vitro* (Noonan *et al*, 1988); however, addition of *cis*-UCA to LC or cultured DC *in vitro* failed to affect their allostimulatory function (Rattis *et al*, 1995; Lappin *et al*, 1997), suggesting that *in vivo*, *cis*-UCA influences APC function indirectly, through induction of secondary mediators. It has been suggested that *cis*-UCA may act through induction of cytokine release, however, studies to examine the *in vitro* effects of *cis*-UCA on cytokine release by keratinocytes (Zak-Prelich *et al*, 2001; Redondo *et al*, 1996) and monocytes (Laihia *et al*, 1994) have failed

to demonstrate alterations in release of cytokines, including IL-1, IL-10, TGF- β and TNF- α , following *cis*-UCA treatment.

The effects of UCA on other cells include suppression of human neutrophil activation *in vitro* by treatment with *cis*- or *trans*-UCA (Kivisto *et al*, 1996), and degranulation of mast cells by *cis*-UCA treatment of human skin organ cultures (Wille *et al*, 1999). In one study, *cis*- but not *trans*-UCA inhibited human *in vitro* NK cell activity in a dose-dependent manner (Gilmour *et al*, 1993); however another study showed that *trans*-UCA suppressed human NK cell function, while *cis*-UCA had no effect (Uksila *et al*, 1994).

It was originally thought that, as *cis*-UCA and histamine are structurally related, *cis*-UCA could act via histamine receptors, and it was demonstrated that treatment of mice with histamine receptor antagonists blocked the suppression of DTH by *cis*-UCA (Norval *et al*, 1990); however, *cis*-UCA does not bind to histamine receptors (Laihia *et al*, 1998). More recently, Hart *et al* (1997) demonstrated that the suppressive effects of *cis*-UCA and histamine on CH responses were not cumulative, suggesting that *cis*-UCA and histamine signalled through the same pathway. *Cis*-UCA does not act directly on mast cells to cause degranulation and histamine release, but is thought to mediate this effect via neuropeptides released from sensory nerve fibres, such as calcitonin gene-related peptide (CGRP) and substance P (Khalil *et al*, 2001). *Cis*-UCA is structurally related to serotonin, and recent evidence suggests that *cis*-UCA may act via the serotonin receptor (S. Ullrich, personal communication).

As discussed in section 1.2.3, UV is strongly absorbed by DNA, leading to the induction of many genes, such as the p53 tumour suppressor gene. UV absorption by DNA is also involved in suppression of immune responses. Induction of DNA strand

breaks by topical application of restriction endonucleases to mouse skin impaired the induction of CH and DTH responses (O'Connor *et al*, 1996; Nishigori *et al*, 1998); treatment with restriction endonucleases also induced TNF- α and IL-10 in murine epidermis, and *in vitro* production of IL-10 and TNF- α by a murine keratinocyte cell line.

Application of the DNA excision repair enzyme T4 endonuclease V, which increases repair of cyclobutyl pyrimidine dimers (CPD), following UV exposure partially protected against UV-induced suppression of CH, and UV-induced morphological effects on epidermal LC (Wolf *et al*, 1995). Treatment of irradiated mice with T4 endonuclease also reduced the number of CPD⁺ cells in the DLN, and restored the antigen presenting activity of these cells, suggesting a role for DNA damage to APC in UV-induced immunosuppression (Vink *et al*, 1996). This was further substantiated by the isolation of APC from the DLN of UV-irradiated, FITC-sensitised mice, which were treated with liposomes containing a photolyase enzyme, followed by photoreactivating light. This treatment decreased the number of cells containing CPD, and restored their antigen presenting ability upon injection into naïve recipients, preventing the induction of transferable regulatory cells in these mice (Vink *et al*, 1997).

Recently, Cruz *et al* (2000) demonstrated that topical application of thymidine dinucleotides to mouse skin caused suppression of CH, and that intradermal injection resulted in activation of the TNF- α gene. These studies imply that UV-induced DNA damage of skin APC may act to alter their ability to present antigen. At the same time, DNA damage by UV in keratinocytes leads to the production of cytokines such as IL-10, IL-6 and TNF- α , which may influence antigen presentation by APC.

1.5.3 - Effects of UVR on APC

It has been suggested by Bacci *et al* (2001) that irradiation of skin transiently inhibits the migration of LC out of the epidermis, thus preventing antigen presentation by these cells in the DLN. However, the majority of studies have demonstrated that UV exposure of skin causes a reduction in the number of human and murine LC, and alterations in the morphology of the remaining LC (Aberer *et al*, 1981; Duthie *et al*, 2000; Kölgen *et al*, 2002). Although *in vitro* UV exposure causes apoptosis of LC (Rattis *et al*, 1998), experiments in mice and sheep indicate that loss of LC from the epidermis following skin irradiation is due to enhanced migration of LC out of the skin, to the DLN (Moodycliffe *et al*, 1992; Dandie *et al*, 2001). Loss of epidermal LC was reversed by antibodies to IL-1 and TNF- α (Duthie *et al*, 2000). Suppression of CH following UV exposure may be related to loss of available APC in the skin at the time of antigen application, however, Noonan *et al* (1984) demonstrated that the waveband dependencies differ for alteration in LC and suppression of CH, implying that the relationship between UV-induced suppression and APC is more complex than this.

UV-induced immunosuppression may arise from alterations in the antigen-presenting function of epidermal cells. T lymphocytes cultured with autologous EC from irradiated human skin showed decreased antigen-induced proliferation compared with EC taken from unirradiated skin (Rasanen *et al*, 1989); the skin was taken 2 h after UV exposure, therefore there was no significant decrease in epidermal LC numbers compared with unirradiated skin. Similarly, *in vitro* exposure of murine epidermal cells to UV prior to antigen-pulsing caused a reduction in their ability to stimulate proliferation of syngeneic immune T cells (Stingl *et al*, 1983). The decrease in antigen

presenting ability of irradiated EC may result from the reduced expression of the co-stimulatory molecules required for T cell activation, leading to anergy of the T cells. *In vitro* UV exposure has been observed to result in decreased upregulation of B7.2 expression by human LC (Rattis *et al*, 1998) and decreased upregulation of B7.1 and B7.2 by murine LC (Denfeld *et al*, 1998). ICAM-1 expression by LC has also been shown to be subject to modulation by UV (Tang and Udey, 1991).

Alternatively, UV-induced suppression of CH responses may result from alteration in the immune responses generated by the irradiated APC. CH is primarily driven by Th1 cells, requiring IL-12 from APC. Simon *et al* (1990) demonstrated that irradiated, antigen-pulsed murine LC were capable of stimulating antigen-specific Th2 cell lines, but not Th1 cell lines. Analysis of DC from lymphoid organs of irradiated mice indicated that following antigen-pulsing these cells produced significantly less IL-12 than DC from unirradiated mice. Incubation of these cells with antigen-specific T cells caused T cell proliferation. However, supernatants from the irradiated-DC cultures contained markedly less IFN- γ (Kitazawa and Streilein, 2000).

Loss of epidermal LC following skin irradiation is accompanied by an influx of inflammatory cells, including neutrophils, macrophages, and monocyte-derived APC (Cooper *et al*, 1993). *In vivo* administration of irradiated mice with CD11b antibody blocked infiltration of the monocyte/macrophage cells into the irradiated skin, and completely restored CH responses (Hammerberg *et al*, 1996); thus, alternative antigen presentation by infiltrating monocyte-derived APC may represent another mechanism by which UV induces suppression in place of an active immune response. These CD11b⁺ infiltrating macrophages have been shown to be potent producers of IL-10 (Kang *et al*, 1994). In addition, a recent study has shown that a high number of the

neutrophils infiltrating irradiated dermis and epidermis stain positive for intracellular IL-4, which may affect the Th1/Th2 balance (Teunissen *et al*, 2002).

1.5.4 - Effects of UVR on keratinocytes

UV exposure of the skin can induce the formation of 'sunburn cells' in the epidermis; these cells are keratinocytes undergoing apoptosis as a result of DNA damage by UV. Irradiation of keratinocytes has also been shown to induce a variety of cytokines, including TNF- α , IL-6 and IL-10; production of these cytokines is thought to result from UV-induced DNA damage (Kibitel *et al*, 1998; Petit-Frere *et al*, 1998; Nishigori *et al*, 1996). Rivas and Ullrich (1992) demonstrated that injection of supernatants from irradiated keratinocytes resulted in suppression of DTH responses in mice; this effect was blocked by anti-IL-10 antibody. Thus, keratinocytes may contribute to UV-induced immunosuppression through production of cytokines that down-regulate immune responses, such as IL-10.

1.5.5 - Effects of UVR on mast cells

Subcutaneous injection of histamine prior to sensitisation suppressed systemic CH responses in mice, and administration of histamine antagonists approximately halved UVB-induced immunosuppression of CH (Hart *et al*, 1997). Treatment with indomethacin partly blocked histamine-induced suppression, suggesting that histamine could suppress immune responses via prostaglandin production. The most likely source of histamine in irradiated mice is mast cells, which also release TNF- α and IL-4. Using mast cell-depleted mice, Hart *et al* (1998) demonstrated the critical role of dermal mast cells in UV-induced immunosuppression. Unlike wild type mice, exposure of these

mice to UVB did not result in systemic suppression of CH responses, nor were these mice susceptible to UV-induced suppression of DTH responses to allogeneic spleen cells. These mice did remain susceptible to local suppression of CH responses by UVR however. Reconstitution of the skin of mast cell-depleted mice with bone marrow-derived mast cell precursors from wild type mice, prior to UV exposure, restored susceptibility to UV-induced suppression of systemic CH responses in these mice.

Mast cells did not degranulate following direct UVB exposure (Danno *et al*, 1988), suggesting that UV causes mast cell degranulation via release of mediators, such as neuropeptides. In sensory-nerve depleted mice, UV exposure failed to induce systemic suppression of a CH response; treatment of wild type mice with a CGRP antagonist reversed UV-induced immune suppression (Garssen *et al*, 1998). As discussed in section 1.5.2, *cis*-UCA may cause mast cell degranulation following UV exposure by causing release of substance P and CGRP from sensory nerves. There is also evidence that release of nerve growth factor (NGF) by keratinocytes in irradiated skin results in neuropeptide release by sensory nerves, leading to mast cell degranulation (Townley *et al*, 2002).

1.5.6 - Effects of UVR on T cells

UV exposure prior to contact sensitisation induces transferable antigen-specific regulatory (also known as suppressor) T cells to haptens applied to the irradiated site (Toews *et al*, 1980; Glass *et al*, 1990). Transfer of DLN cells from UV-irradiated, fluorescein isothiocyanate (FITC)-sensitised mice to naïve recipients, followed by challenge of the recipients with FITC, resulted in the induction of transferable regulatory cells in the spleens of these mice, which was dependent on the presence of

Thy1.2⁺ and CD4⁺ cells in the DLN cell suspensions (Saijo *et al*, 1995). Injection of FITC-bearing DLN cells from unirradiated mice into UV-irradiated recipients, followed by challenge of the recipients with FITC, failed to induce a CH response, and resulted in formation of regulatory T cells; this effect was blocked by treatment of the UV-irradiated recipients with anti-TNF- α (Saijo *et al*, 1995). Thus, UV-induced transferable regulatory T cells can occur in the absence of altered antigen presentation, and may depend on TNF- α .

To further characterise these UV-induced regulatory cells, hapten-specific cell lines and clones were established from the LN of irradiated and unirradiated FITC-sensitised mice. These cells were CD4⁺ and TCR $\alpha\beta$ ⁺, and proliferated in response to APC taken from sensitised animals; however, whereas cell lines from unirradiated mice were Th1 like, producing large amounts of IFN- γ , but little IL-4 or IL-10, cloned cells from irradiated mice produced IL-10, but no IL-4 or IFN- γ , and blocked APC functions and IL-12 production *in vitro* (Shreedhar *et al*, 1998).

More recently, Schwarz *et al* (2000) demonstrated that depletion of CTLA-4⁺ cells prior to injection of LN cells from irradiated, dinitrofluorobenzene (DNFB) mice into naïve recipients prevented the transfer of suppression to these mice. Additionally, UV-induced tolerance and transfer of suppression of CH could be prevented by *in vivo* injection of anti-CTLA-4. CTLA-4 is able to ligate B7.1 and B7.2, but unlike CD28, CTLA-4 is thought to provide a regulatory, rather than a co-stimulatory signal, blocking T cell activation (Walunas *et al*, 1996). *In vitro* stimulation of CTLA-4⁺ cells from irradiated, sensitised mice results in secretion of high levels of IL-10, TGF- β and IFN- γ , with low level of IL-2, and no IL-4 (Schwarz *et al*, 2000). Additionally, treatment of

UV-CTLA-4⁺ cell recipients with anti-IL-10 prevented transfer of tolerance to these mice.

Moodycliffe *et al* (2000) investigated the role of NKT cells in UV-induced suppression of DTH responses to *Candida albicans*. NKT cells express intermediate amounts of T cell receptor molecules, coexpress surface antigens normally found on NK cells, have a restricted T cell receptor usage, are CD4⁺, restricted by CD1, and secrete IL-4. While transfer of T cells from irradiated, *C. albicans* immunised mice to *C. albicans*-sensitised syngeneic recipients resulted in suppression of the DTH response, removal of NKT cells from the T cell suspension abrogated the suppression.

The results of Schwarz *et al* (2000) and Moodycliffe *et al* (2000) indicate that different types of regulatory T cells are involved in UV-induced suppression of CH and DTH responses. The cytokines produced by these regulatory cells point to a general shift away from Th1 responses towards the production of Th2 cytokines following UV exposure.

1.6 - UV-induced suppression of tumour immunity

1.6.1 - UV exposure and growth of UV-induced tumours

As described in section 1.4.1, the majority of UV-induced tumours in mice are rejected upon transplantation to immunocompetent hosts. Kripke and Fisher (1976) demonstrated that a short course of UV irradiation, as little as two weeks, made mice susceptible to challenge with a UV-induced tumour, which grew progressively in these mice. This result was confirmed by Daynes *et al* (1977). UV exposure did not affect established anti-tumour responses however, such that irradiation of mice previously

immunised against a UV-induced tumour did not alter their ability to reject tumour challenge (Kripke and Fisher, 1976). Parabiosis of two mice, one UV-irradiated and one untreated, followed by tumour implantation, resulted in progressive tumour outgrowth in both the irradiated and unirradiated mice; this suggested that prior UV exposure resulted in an active, systemic suppression (Fisher and Kripke, 1977). Splenic lymphocytes from tumour-bearing mice exhibited tumour-specific cytotoxicity *in vitro*; irradiation prior to tumour-implantation abrogated this *in vitro* cytotoxicity. Transfer of lymphoid (spleen and lymph node) cells from UV-irradiated to x-irradiated mice resulted in progressive growth of implanted tumours in the recipient mice, indicating the immunological nature of this suppression. (Fisher and Kripke, 1977).

The role of UV-induced immunosuppression in photocarcinogenesis in humans is less easy to establish. The increased incidence of skin cancer in transplant patients taking immunosuppressive drugs, that have high levels of sun exposure, may result from the greater sun exposure causing more mutagenic events. However, it has been suggested that in those patients with high sun exposure, UV-induced immunosuppression may exert an additive effect on their already immunosuppressed state (Boyle *et al*, 1984; Caforio *et al*, 2001). Yoshikawa *et al* (1990) tested the effect of low dose UVB exposure on CH responses in NMSC patients and healthy controls; more than 90% of skin cancer patients demonstrated susceptibility to UVB-induced suppression, compared with only 40% of controls. This implied that skin cancer patients are more susceptible to UV-induced immunosuppression, which may allow outgrowth of antigenic UV-induced tumours.

1.6.2 - Regulatory cells and UV-induced suppression of anti-tumour responses

The role of T 'suppressor' cells in outgrowth of primary UV-induced tumours was shown by injection of T-cell enriched lymphoid cells to mice during photocarcinogenesis; mice receiving T cell from UV-irradiated donors developed more tumours than mice receiving T cells from normal mice (Fisher and Kripke, 1982).

Whereas immune responses mounted against UV-induced antigenic tumours showed tumour specificity, UV-induced 'suppressor' cells conferred suppression against more than one UV-induced tumour (Kripke and Fisher, 1976). This, along with the establishment of a cloned UV-induced suppressor T cell line that could inhibit immune responses against a variety of UV-induced tumours (Roberts, 1986), suggested that UV-induced 'suppressor' cells recognise a common antigen found on UV-induced tumours.

More recently, attempts to characterise the UV-induced T regulatory cells that control outgrowth of tumours in irradiated mice have shown two types of T cell to be important. Beissert *et al* (1999) used transgenic mice that were unable to signal via the B7/CD28-CTLA-4 co-stimulatory pathways; chronic UV exposure of these mice resulted in the development of fewer tumours than arose in control wild-type mice. T cells isolated from these transgenic mice and stimulated *in vitro* produced significantly less IL-4, but more IFN γ than wild-type mice, suggesting that these mice have an impaired Th2 response, and increased Th1 immunity. Thus, UV-induced alteration of the Th1/Th2 balance may be responsible for suppression of anti-tumour responses seen following UV exposure.

In a different approach, Moodycliffe *et al* (2000) examined the role of NKT cells (described in section 1.5.6) in UV-induced suppression of anti-tumour responses.

Reconstitution of x-irradiated mice with NKT cells from irradiated, but not unirradiated mice resulted in progressive outgrowth of a regressor UV-induced tumour in these mice. However, reconstitution with NKT cells failed to suppress the rejection of a regressor chemically-induced tumour, suggesting that UV-induced NKT cells recognise UV-induced tumours as a class, perhaps by the common antigen previously shown to be recognised by UV-induced 'suppressor' cells (Kripke and Fisher, 1976; Roberts, 1986).

1.6.3 - Antigen presentation and UV-induced suppression of anti-tumour responses

As discussed in section 1.5.3, UV exposure has numerous effects on epidermal APC, including a reduction of their numbers and antigen presenting activity. As described in section 1.4.3, mice immunised with tumour antigen-pulsed syngeneic EC demonstrated protective immunity to subsequent challenge with live tumour cells, and were also able to mount a DTH response to killed tumour cells. UV irradiation of the EC prior to injection into mice abrogated their ability to induce protective tumour immunity, and to elicit a DTH response to tumour cells (Grabbe *et al*, 1992). Use of anti-TNF- α antibodies suggested that this effect of UV was not dependent on TNF- α .

Studies of chemicals that cause promotion of tumour outgrowth have shown that these chemicals can cause depletion of LC from the epidermis (Muller *et al*, 1985; Halliday *et al*, 1987); therefore, loss of LC from the skin following UVB may affect the induction of anti-tumour responses. A study by Sluyter and Halliday (2000) found that following exposure of mice to a single high dose of UV, reduction in epidermal LC did not occur until at least 4 days after irradiation, whereas increased outgrowth of implanted tumours only occurred if the tumours were inoculated less than 4 days after

irradiation. Although they failed to find a correlation between LC number and enhanced tumour outgrowth, this study indicated that UV effects on tumour outgrowth were associated with infiltration of inflammatory cells, including macrophages, into the epidermis. As discussed in section 1.5.3 MHC class II⁺, CD11b⁺ macrophages present in UV-irradiated epidermis are known to produce IL-10, and can induce tolerance to a contact sensitizer (Kang *et al*, 1994; Hammerberg *et al*, 1996). It is possible that alternative antigen presentation by these cells leads to the induction of regulatory cells; alternatively, production of IL-10 by these cells may affect the induction of an effective anti-tumour immune response.

1.6.4 - NK cells and UV-induced suppression of anti-tumour responses

One study assessed the activity of NK cells during photocarcinogenesis of nude mice (Toda *et al*, 1986). Chronic UV exposure resulted in significantly suppressed activity, measured *in vitro*, of splenic NK cells from irradiated mice; this was apparent from week 15 of UV exposure, and continued to decrease. Tumours first appeared at 19 weeks. It is not clear whether suppression of NK activity was important for tumour appearance in these mice; additionally, while NK cells may have a role in protection from photocarcinogenesis in nude mice, which lack T cells, suppression of NK cell activity by UV might not be an important mechanism of suppression in immunocompetent mice.

1.6.5 - Mast cells and UV-induced suppression of anti-tumour responses

Two studies using hairless mouse models suggested that the mast cell product histamine is involved in photocarcinogenesis. Feeding mice cimetidine, a histamine

type 2 receptor antagonist, during chronic UV exposure reduced the number of tumours that appeared (Matheson and Reeve, 1991). Feeding mice indomethacin, which blocks prostaglandin production, during chronic UV exposure, also protected against later tumour development (Reeve *et al*, 1995).

A recent human study found that BCC patients had a higher median dermal mast cell prevalence than healthy controls (Grimbaldeston *et al*, 2000). The authors suggested that UV exposure initiates immunosuppression through these dermal mast cells, resulting in a permissive environment for BCC development.

1.6.6 - *Cis*-UCA and UV-induced suppression of anti-tumour responses

Evidence from a small number of animal studies has pointed to a role for *cis*-UCA in outgrowth of UV-induced tumours. During photocarcinogenesis of hairless mice by daily exposure to a MED of UV, Reeve *et al* (1989) demonstrated that topical application of UCA prior to each irradiation increased both the number of tumours produced, and the degree of malignancy of those tumours. In mice exposed to UV for 10 weeks, and later treated with the tumour promoter croton oil to reveal latent tumours, mice that had received UCA topically prior to each irradiation were found to have an increased number of latent tumours. Thus, UCA treatment appeared to promote the survival of UV-initiated cells, perhaps by *cis*-UCA-induced suppression of local immune responses to the altered cells.

More recently, Beissert *et al* (2001) chronically exposed mice to UV three times a week for 6 months; before each UV treatment, mice were injected with a monoclonal antibody specific for *cis*-UCA, or an irrelevant control antibody. The probability of development of cutaneous malignancies was significantly reduced in the mice treated

with the anti-*cis*-UCA antibody, suggesting that *cis*-UCA formed following UV exposure has a role in photocarcinogenesis.

Cis-UCA may be important during photocarcinogenesis due to effects on presentation of tumour antigens. As described in section 1.4.3, mice immunised with tumour antigen-pulsed syngeneic EC demonstrated protective immunity to subsequent challenge with live tumour cells, and were also able to mount a DTH response to killed tumour cells. Incubation of EC in *cis*-UCA prior to tumour antigen pulsing abrogated their ability to induce protective tumour immunity and to elicit a DTH response in tumour immune mice; incubation with *trans*-UCA had no effect (Beissert *et al*, 1997). The effect of *cis*-UCA on tumour immunity could not be reversed by addition of a histamine antagonist, or indomethacin, which blocks prostaglandin production, to the incubation medium (Beissert *et al*, 1997). However, addition of IL-12 to the *cis*-UCA incubated EC restored their ability to induce protective tumour immunity, and to elicit a DTH response in tumour immune mice (Beissert *et al*, 2001).

Attempts to investigate the role of UCA in human skin cancers have focussed on comparing the amounts of UCA in the skin of cancer patients and controls. A Danish study measured the UCA concentration in skin that is regularly exposed to UV (forehead) or not exposed to UV (buttock); no difference in total UCA or *cis*-UCA concentration in the skin of patients with a past history of BCC or malignant melanoma compared with healthy controls was found (de Fine Olivarius *et al*, 1998). In a similar study conducted in Italy, the concentration of UCA in the skin of NMSC patients was measured during the summer and winter months. No difference in the concentration of UCA or percentage of *cis*-UCA in the buttock skin of NMSC patients compared with healthy controls was seen; however, the percentage of *cis*-UCA in arm skin was lower

in NMSC patients than healthy controls during the summer months, possibly due to the patients avoiding sun exposure (de Simone *et al*, 2001). Additionally, the production of *cis*-UCA in skin following UV irradiation has been measured. In one study, a slight increase in *cis*-UCA production was seen in the skin of BCC and melanoma patients compared with healthy controls (de Fine Olivarius *et al*, 1998). A second study showed no difference in *cis*-UCA production between controls and BCC patients, and a slight decrease in *cis*-UCA production in the skin of melanoma patients (Snellman *et al*, 1999).

1.6.7 - UV-induced immunosuppression in melanoma

Evidence of the immunogenicity of melanomas, and the development of a murine model of melanoma, has led to investigations of the role of UV-induced immunosuppression in melanoma outgrowth in mice. As seen with other UV-induced tumours, UV exposure of mice prior to injection with melanoma cells resulted in an increased incidence and size of tumours compared with those in mice not irradiated prior to melanoma cell injection (Romerdaal *et al*, 1988). Injection of melanoma cell into the unirradiated skin of irradiated mice had no effect on tumour outgrowth however, indicating that UV exposure acted locally to alter tumour outgrowth in irradiated skin. UV irradiation of x-irradiated, athymic or Thyl⁺ cell-depleted mice prior to injection of melanoma cells had no effect on tumour outgrowth in these mice, suggesting that immunological mechanisms have a role in UV-enhanced melanoma cell outgrowth (Donawho and Kripke, 1991). Donawho *et al* (1996) demonstrated that tumour rejection in melanoma-immune mice is suppressed when they are challenged in a UV-irradiated site, but not in unirradiated skin following UV-exposure. Melanoma

growth could be inhibited in normal mice if melanoma cells were mixed with lymphoid cells from tumour-immune mice prior to injection; however, injection of this mixture into previously irradiated skin resulted in tumour outgrowth. Analysis of tumour infiltrating lymphocytes demonstrated a significant decrease in numbers of CD8⁺ cells in tumours growing in a UV-irradiated site. From these experiments, UV exposure appears to suppress the number and activity of effector cells in the irradiated site.

1.6.8 - Sunscreens and UV-induced suppression of anti-tumour responses

A study by Roberts and Beasley (1997) found that application of high sun protection factor sunscreens prior to each irradiation protected against enhanced tumour outgrowth seen in mice exposed to solar-simulating UV for 6 weeks prior to tumour transplantation of NMSC. In this and other studies (Morison, 1984; Gurish *et al*, 1981), sunscreen application also prevented the induction of transferable tumour-specific regulatory T cells in UV-irradiated mice. In contrast, Wolf *et al* (1994) demonstrated that application of low sun protection factor sunscreens, which blocked UV-induced oedema, did not protect against UV enhanced outgrowth of melanoma cells. However, it may be that while the sunscreens protected the mice from oedema-inducing UV doses, the mice still received sufficient UV to cause suppression of anti-tumour responses.

1.7 - Hypotheses tested in this investigation

1.7.1 - Multiple exposures of UVB or UVA irradiation suppress immune responses

Use of an experimental protocol with multiple UV exposures bears more resemblance to the sunlight exposure patterns of humans, than the single, often high

dose of UV that the majority of studies on UV-induced immunosuppression have used. In the present study, the effect of exposure of mice to a 3 week suberythematous UV protocol on various immune parameters was assessed. CH responses, epidermal LC number and dendritic cell numbers in LN draining the irradiated sites were measured following broad-band UVB exposure of mice, to determine whether multiple UV exposures have similar effects to a single exposure on these immune responses. The effects of UVA and narrow band UVB (TL01) exposure over the 3 week period on CH and epidermal LC number were compared with that of broad-band UVB exposure, to assess the waveband dependence of immunosuppression following repeated UV exposure.

1.7.2 - UVA-I irradiation can contribute to tumour outgrowth by suppressing immune responses to tumours

UVB exposure is thought to have a dual role in photocarcinogenesis, inducing DNA damage that gives rise to neoplastic cells, and suppressing immune responses that might otherwise destroy these transformed cells. Although less effective than wavelengths within the UVB range, wavelengths above 340 nm (UVA) cause carcinogenesis in the hairless mouse. In the present study, the hypothesis that UVA can suppress immune responses to skin tumours was tested using a mouse fibrosarcoma (FSA) model. Mice were irradiated for a period of 3 weeks, and FSA cells were then injected subcutaneously. The effect of UVA, and broad-band and narrow-band UVB on tumour cell outgrowth was measured. The dose dependency of UV-enhanced tumour outgrowth was also assessed.

1.7.3 - UVB exposure suppresses systemic immune responses in tumour-bearing mice

Regulatory T cells are present in the lymphoid organs of irradiated, tumour-bearing mice. Therefore these mice are likely to have suppressed immune responses to the tumour, such that control of tumour growth by the immune system is lost. An attempt was made to develop *in vivo* and *in vitro* assays to measure tumour immunity in tumour-bearing mice. Tumours were induced by subcutaneous injection of FSA cells. Changes in proliferation and surface marker expression of LN cells from irradiated and unirradiated tumour-bearing mice were assessed to try to understand the mechanisms by which UV exposure of mice results in increased tumour outgrowth.

1.7.4 - *Cis*-UCA is the chromophore that initiates UV-induced suppression of immune responses to tumour cells.

The hypothesis that *cis*-UCA is involved in UV-induced suppression of anti-tumour responses was tested in two ways. First, mice were treated for a period of 3 weeks with *cis*-UCA prior to injection of FSA cells, and the growth of the resulting tumour measured. Second, mice exposed to UVB for 3 weeks were injected with anti-*cis*-UCA antibody prior to each irradiation; these mice were then injected with FSA cells and tumour growth monitored.

Chapter 2

Materials and Methods

2.1 General

2.1.1 - Mice

C3H/HeN (H-2k) mice were obtained from the Medical Faculty Animal Area, University of Edinburgh; they were housed in a room where ambient light was regulated on a 12 h light/dark cycle and had free access to food and water. Room lights were shielded such that any contaminating UV wavelengths were filtered out. Female mice aged 6-10 weeks were used unless otherwise stated.

2.1.2 - Growth medium and supplements

RPMI-1640 (Gibco, BRL) was supplemented with 100 I.U./ml penicillin, 200 mg/ml streptomycin, 2 mM L-glutamine, 100 µg/ml gentamicin, 1.5 µg/ml fungizone and 10% heat-inactivated foetal calf serum (FCS; Gibco, BRL) designated RPMI-FCS throughout. In some experiments RPMI-FCS was supplemented with 10 mM HEPES (Sigma, Poole, Dorset) and 2×10^{-5} M β -mercaptoethanol (RPMI-HEPES).

2.1.3 - Cells

Fibrosarcoma (FSA) cells were obtained from Professor William McBride (University of California, Los Angeles); they are moderately immunogenic and are derived from a fibrosarcoma that was induced in C3H mice by methylcholanthrene treatment (Suit and Kastellan, 1970). Cells were cultured in RPMI-FCS; they were harvested using a 0.1% (v/v) trypsin/ 0.04% (w/v) versene solution and the number

of viable cells determined by trypan blue exclusion. For injection into mice, cells were washed once in RPMI-FCS, then resuspended in Hanks' balanced salt solution (HBSS), supplemented with 20 µg/ml deoxyribonuclease I (Sigma) (HBSS-DNase).

2.1.4 - UV sources and exposure

Mice were shaved on the back at least 24 h before the first irradiation and again 24 h before the final irradiation. Control mice were shaved but not irradiated. Mice were placed in a Perspex box with no more than 4 mice per box to avoid shielding by littermates. Mice were irradiated twice a week for 3 weeks, unless otherwise stated. Three UV sources with different emission spectra were used (Figure 2.1) and their outputs determined using a filtered photodiode meter, calibrated against measurements made with a UV-visible spectroradiometer (model 742, Optronic laboratories) across the spectral range 250-400 nm. The broad-band UVB source was provided by two Philips TL-20W/12 bulbs with an output range of 270-350 nm, peak 305 nm, emitting 80 mW/cm². The tube to target distance was 16 cm. One minimal erythral dose (MED) for C3H/HeN mice under these conditions was 1500 J/m². The narrow-band UVB source was a Philips TL01 lamp, which emitted predominantly (51%) at 310-312nm; the total irradiance was 200 µW/cm². One MED for C3H/HeN mice was 10,000 J/m². For UVA exposure, a Dr. Hoenle Bluelight lamp was used. This had an output range 315-400 nm, emitting 3.3 mW/cm²; for UVA-I exposure, contaminating UVB was filtered out using Cation X (provided by Dr. F de Gruijl, Utrecht) at 0.1 mg/ml in distilled water, at a depth of 1cm in a tray made using window glass 2 mm deep. One MED for C3H/HeN mice under these conditions was 500,000 J/m².

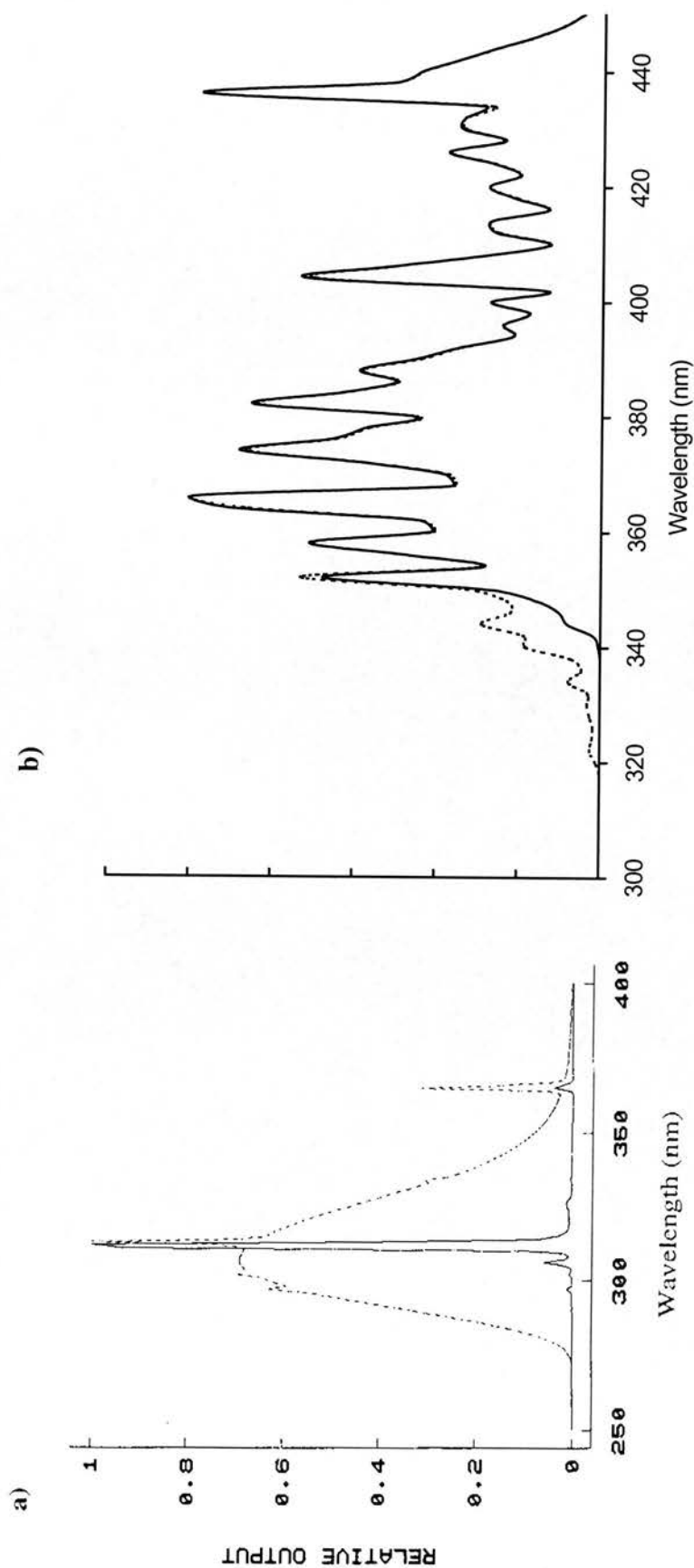


Figure 2.1. The spectral emission of the three UV sources. a) the broad-band (TL20W/12) UVB (dotted line) and narrow-band TL01 UVB (solid line) and b) the UVA source (the dotted line indicates wavelengths absorbed by Cation-X).

2.1.5 - Urocanic acid isomers

Trans-urocanic acid (*trans*-UCA; 4-imidazoleacrylic acid) was purchased from Sigma. *Cis*-UCA was prepared by irradiation of *trans*-UCA, followed by purification by thin-layer chromatography to give a greater than 99% pure preparation, as described previously (Norval *et al*, 1989). *Cis*-UCA was provided by Dr. J Crosby, University of Bristol.

2.1.6 - Recombinant IL-12 injection

Recombinant mouse IL-12 (rIL-12) was purchased from Serotec (Oxford, UK). Mice received 50 ng of rIL-12 in 200 µl sterile, endotoxin-free PBS, by intraperitoneal injection, 4 h after each irradiation.

2.2 Hypersensitivity responses

2.2.1 - Contact Hypersensitivity response to oxazolone

Oxazolone (50 µl of a 1% (w/v) solution in 4:1 acetone:olive oil; 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one, Sigma) was applied to the shaved dorsal skin; 50 µl vehicle only was applied for negative controls. Mice were tailmarked 8 days later; they were then anaesthetised with halothane and the thickness of their ears measured using an engineer's micrometer (Mitutoyo Corporation, Japan). A challenge dose of 0.5% oxazolone in 25 µl vehicle was applied to both ears of all mice. Ear swelling was measured 24 h later, and the mean ear swelling and standard error of the mean (SEM) calculated for each group.

2.2.2 - Delayed hypersensitivity response to FSA cells

FSA cells (10^4 in 100 μ l HBSS-DNAse) were injected either subcutaneously in 4 dorsal sites, or intravenously into the tail vein of normal mice. Control mice received 100 μ l HBSS only, either subcutaneously or intravenously. Mice were tailmarked 14 days later and ear thickness measured with an engineer's micrometer. They were then challenged intrapinnally with FSA cells (10^5 in 10 μ l HBSS) which had been inactivated by exposure to 18,000 J/m² broad-band UVB (TL20W/12) directly prior to injection. Ear swelling was measured 24 h later and the mean ear swelling and SEM calculated for each group.

2.3 - Langerhans cell counts

The number of Langerhans cells in the epidermis of UV-irradiated and control mice was determined by staining for adenine triphosphatase (ATPase) using adenine diphosphate as a substrate (Chaker *et al*, 1984). Mice were killed by cervical dislocation 24 h after the final irradiation, and their ears removed into ice-cold PBS. The dorsal surface was separated from the lower surface using forceps, and floated epidermal side down for 2 h at 37°C in 0.76% (w/v) tetrasodium ethylenediamine tetraacetic acid (EDTA; Sigma). After washing 3 times in PBS, epidermal sheets were removed using a scalpel blade, and fixed by floating in sodium cacodylate/formaldehyde buffer [6.85% (w/v) sucrose, 1.6% (w/v) sodium cacodylate, in distilled water, with 10% (v/v) formaldehyde solution (Merck Eurolab Etd, Lutterworth, Leic.)] for 1 h at 4°C. Sheets were washed in PBS again, then stained with ADP-lead [0.12% (w/v) MgSO₄, 5% (w/v) D-glucose, 0.06% (w/v) ADP (Sigma), 0.12% (w/v) Pb(NO₃)₂] in Tris/Mal buffer [3.025% (w/v) Tris buffer

salt, 2.9% (w/v) maleic acid, 1% (w/v) NaOH; pH 7.3] for 70 min at 37°C. Sheets were then rinsed 3 times in Tris/mal buffer:distilled water 2:3. The stain was developed in 2% (w/v) ammonium polysulfide (20 min at room temperature); sheets were then rinsed in tap water and mounted onto a glass slide under a cover slip in 50% (v/v) glycerol. The number of ATPase⁺ cells in 10 fields per epidermal sheet was counted (1 field = 0.1 mm²), with a minimum of 4 sheets per group.

2.4 Injection of tumour cells

2.4.1 - Injection of FSA cells in UV exposed skin

Mice were anaesthetised with halothane 24 h after the final irradiation and 7.5×10^4 fibrosarcoma (FSA) cells in 100 µl HBSS-DNAse were injected subcutaneously into 4 sites in the irradiated back skin. Mice were monitored daily for the appearance of tumours; once the tumours were a measurable size two bisecting diameters were measured using a ruler. The tumours were not a uniform shape, therefore an arbitrary measurement of tumour size was calculated by multiplying together the values for the two bisecting diameters; the sum of the tumour sizes for a single mouse was calculated to give the total tumour area for that mouse. The tumour growth was measured for a period of 4 weeks, or until the tumours either showed ulceration or reached a diameter of 10 mm, at which point the mice were killed by cervical dislocation.

2.4.2 - Injection of FSA cells in unirradiated skin of irradiated mice

Mice were anaesthetised 24 h prior to the final irradiation and their ventral hair shaved; 24 h after the final irradiation they were anaesthetised again and 7.5×10^4

FSA cells injected subcutaneously into 4 ventral sites. The resulting tumours were measured as already described.

2.4.3 - Intravenous injection of FSA cells

FSA cells were resuspended to 10^6 cells/ml in HBSS-DNase and 100 μ l injected intravenously into the tail vein. Mice were killed 14 days later and their lungs removed into Bouin's fluid; following fixation for 24 h the number of lung colonies was counted by eye.

2.5 Proliferation and phenotyping of draining lymph node cells

2.5.1 - Collection and preparation of draining lymph node (DLN) cells

Lymph nodes draining the dorsal skin (unless otherwise stated) were collected and pooled in RPMI-HEPES. Single cell suspensions were prepared by mechanical disaggregation through a nylon cell strainer (Becton Dickinson, Bedford, USA) and washed once in RPMI-HEPES. Viable cells were counted by trypan blue exclusion. The number of cells per lymph node for a single group of mice was calculated using the following formula:

$$\frac{\text{Cell count per ml} \times \text{Total volume of cell suspension}}{\text{Number of LN collected}}$$

2.5.2 - Spontaneous proliferation of DLN cells

Cells were resuspended to 2×10^6 cells/ml, and 200 μ l seeded into wells of a 96-well round-bottom culture plate (Iwaki, Asahi Techno Glass, Japan). Cells were radioactively pulsed by adding 0.7 μ Ci 3 H-methyl thymidine (specific activity 2.0



Ci/mmol; Amersham Life Science, UK) in 10 μ l RPMI-HEPES per well and incubated for 24 h at 37°C in a humid atmosphere of 5% CO₂ in air. Cells were harvested onto filter mats and ³H-thymidine incorporation measured in counts per minute using a scintillation counter (Canberra Packard, Zurich, Switzerland).

2.5.3 - Proliferation of DLN cells in response to mitogens

Cell suspensions were prepared as described in section 2.4.1 and 100 μ l (2×10^5 cells) seeded into a 96-well round bottom plate. The mitogens concanavalin A (Con A; Sigma) or phytohaemagglutinin (PHA; Sigma), were dissolved in RPMI-HEPES and added to the cells in 100 μ l volumes to give a final concentration of 2.5 μ g/ml. Alternatively, an antibody to murine CD3 (Serotec) was dissolved similarly and added to the cells at a final concentration of 1 μ g/ml. The cultures were incubated as previously described for 48 h or 72 h; cells were pulsed with 0.7 μ Ci/well ³H-methyl thymidine 8 h prior to harvesting.

2.5.4 - Proliferation of DLN cells in response to tumour antigens

Confluent monolayers of FSA cells in 75 cm² tissue culture flasks were rinsed in PBS and harvested using a sterile cell scraper (Merck Eurolab Ltd.). Glycine extracts of tumour cell antigens (TA) were prepared from FSA cells using the method of Booth *et al* (1979). Briefly, cells were washed once in PBS and resuspended in glycine buffer, prepared from five parts 0.1 M aqueous solution of glycine and three parts 0.1 M aqueous sodium hydroxide (equivalent to 0.5 ml of buffer per culture flask of cells). The cells were disrupted by three freeze/thaw cycles, with vigorous agitation and ultrasonication (1 min) between each cycle. The cellular debris was

removed by centrifugation at 800 g for 10 min, and the final supernatant stored in small aliquots at -70°C . The protein concentration of the extract was determined by the Lowry method (Lowry *et al*, 1951).

Lymph node and spleen cell suspensions were prepared as described in section 2.5.1 and $100\ \mu\text{l}$ (2×10^5 cells) seeded into a 96-well round bottom plate. Glycine extracted TA were diluted in RPMI-HEPES and $100\ \mu\text{l}$ added to the cells, to give final concentrations of 0.2-200 μg protein/ml. Cells were incubated for 24-96 h, and proliferation measured by thymidine incorporation as described in section 2.5.3. To test whether the glycine extraction buffer affected proliferation of the cells, LN and spleen cells from a tumour-bearing mouse were cultured for 96 h in the presence of TA-free glycine buffer diluted in RPMI-HEPES (Figure 2.2). The glycine extract was toxic at the highest concentration used, but otherwise had little effect on the proliferation of the cells.

2.5.5 - Measurement of IL-10 production by ELISA

Lymph node cell suspensions were prepared (section 2.5.1) to a concentration of 10^7 cells/ml and 1 ml seeded into the wells of a 24-well tissue culture plate (Iwaki). Supernatants were collected at 24, 48 and 120 h of culture and stored at -70°C until assayed for IL-10 content. IL-10 was assayed using a Quantikine mouse IL-10 immunoassay kit (R&D Systems) according to the manufacturer's instructions. For each assay a standard curve was created by addition of standards to a row of wells; these ranged from 0-1000 pg/ml. Standards and samples were diluted 1/2 in diluent buffer, $100\ \mu\text{l}$ added per well, and incubated for 2 h at RT. Control wells received

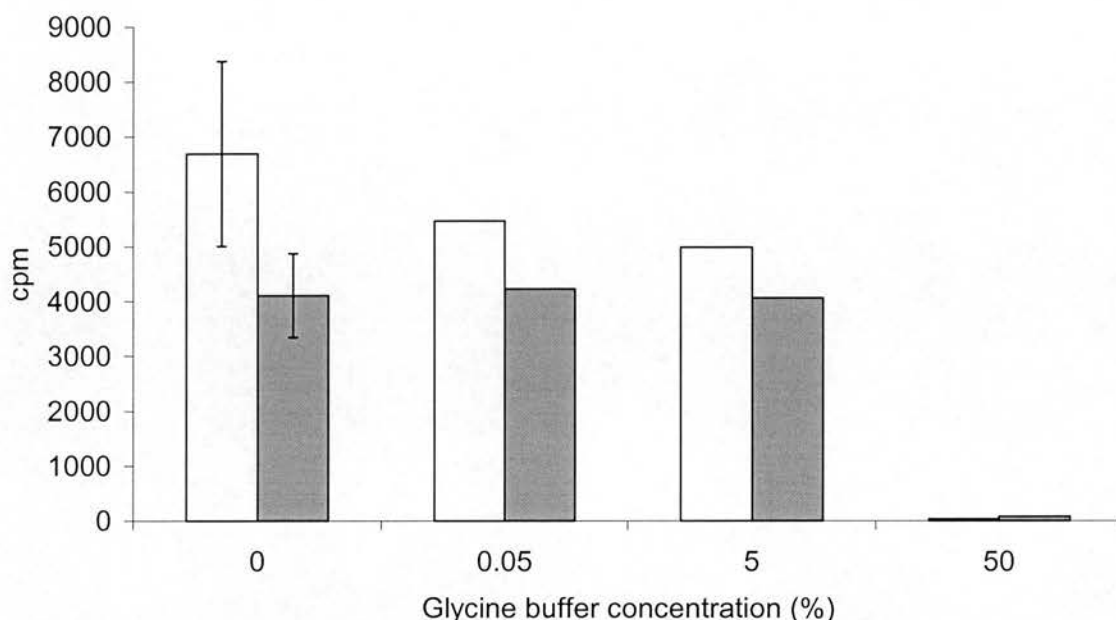


Figure 2.2. Proliferation of immune cells cultured in varying concentrations of glycine buffer. Cells from LN draining the tumour site (clear columns) or the spleen (filled columns) were cultured for 96 h in medium alone (0), or with varying concentrations of glycine buffer (0.05-50 % in medium); ^3H -Thymidine was added for the final 8 h of culture. The cells were taken from tumour-bearing mice that had received 7.5×10^4 FSA cells in 4 dorsal sites 20 days previously. Error bars show the SEM of 3 replicate wells (only shown for cells incubated in medium alone).

diluent buffer only. After washing 4 times in wash buffer, 100 μl of horse radish peroxidase (HRP)-conjugated anti-mouse IL-10 antibody was added to each well for a further 2 h at RT. Following a further wash, 100 μl stabilized chromagen was added to each well for 20 min in the dark, before the addition of stop solution. Absorbance at 450 nm of each well was read on a plate reader (MR 700 Plate Reader, Dynatech) blanked against the control wells. The minimum detectable dose of mouse IL-10 was 4.0 pg/ml, according to the manufacturers.

2.5.6 - Phenotyping of DLN cells by flow cytometry

Monoclonal antibodies raised in rat, recognising Thy1.2, CD8, CD4, CD11b, Ia and CD86 (B7.2), and negative control antibodies (isotypes IgG2a and IgG2b) were all purchased from Serotec and titrated to determine the optimal concentration (shown in Table 2.1). Lymph node cell suspensions were prepared as described in section 2.4.1 and 5×10^5 cells incubated on ice for 45 min with 500 μ l control antibody or the primary monoclonals listed in Table 2.1. The cells were washed once with 1 ml RPMI-HEPES, resuspended in RPMI-HEPES and incubated with an affinity purified F(ab')₂ goat anti-rat IgG-FITC conjugate (100 μ l of 1/100 dilution; Serotec) for 45 min on ice. Cells were washed again, resuspended and fixed in 1 ml 10% formol saline and analysed using a Coulter XL Flow cytometer. Cells were identified first

Specificity	Clone	Isotype	Concentration
Human CD8 (control)	YTH862.2	IgG2b	1/500
Serotec negative control	Not known	IgG2a	1/200
Murine Thy1.2	FF-10	IgG1	1/1000
Murine CD4	YTS191.1	IgG2b	1/1000
Murine CD8	YTS105.18	IgG2a	1/500
Murine CD11b (CR3)	5C6	IgG2b	1/500
Murine CD86 (B7.2)	RMMP-2	IgG2a	1/200
Murine Ia	Ye2/36hlk	IgG2a	Neat (30 μ l)

Table 2.1. Rat Monoclonal antibodies to mouse lymphocyte cell surface markers.

using forward scatter and side angle light scatter to quantify their size and granularity. Gates were placed around the entire cell population and around cells with low side scatter and forward scatter (lymphocytes) and around larger, more granular cells (Figure 2.3a). The events within each region were displayed on histograms of log fluorescence intensity (x-axis) against cell count (y-axis). Isotype controls were routinely set at 1 % and a minimum of 20,000 events was accumulated in the region around the entire cell population.

2.5.7 - DC enrichment and phenotyping

Mice were killed by cervical dislocation 48 h after the final UV irradiation or were unirradiated (8 mice per group). Their auricular, axillary and inguinal LN were collected and pooled in RPMI-HEPES. Single cell suspensions were prepared as described in section 2.4.1. The LN cell suspensions were enriched for DC using the method of Macatonia *et al* (1989). Briefly, 4×10^7 LN cells in 8 ml medium were underlaid with 2 ml metrizamide (14.5 % in RPMI-HEPES, Sigma, UK). Following a 15 min centrifugation at 600g, the interface layer was collected, washed and resuspended in a minimal amount of medium. The number of DC was assessed by morphological examination by light microscopy; at least 5 counts for each group were made, and the mean number of DC per LN was calculated. Using this method, 55-70% of the cells in the DC-enriched suspension were DC. Expression of the surface markers Ia and CD86 was determined by flow cytometry (10^4 cells per antibody) as described in section 2.4.6. The scatter profile of the DC enriched population is shown in Figure 2.3b.

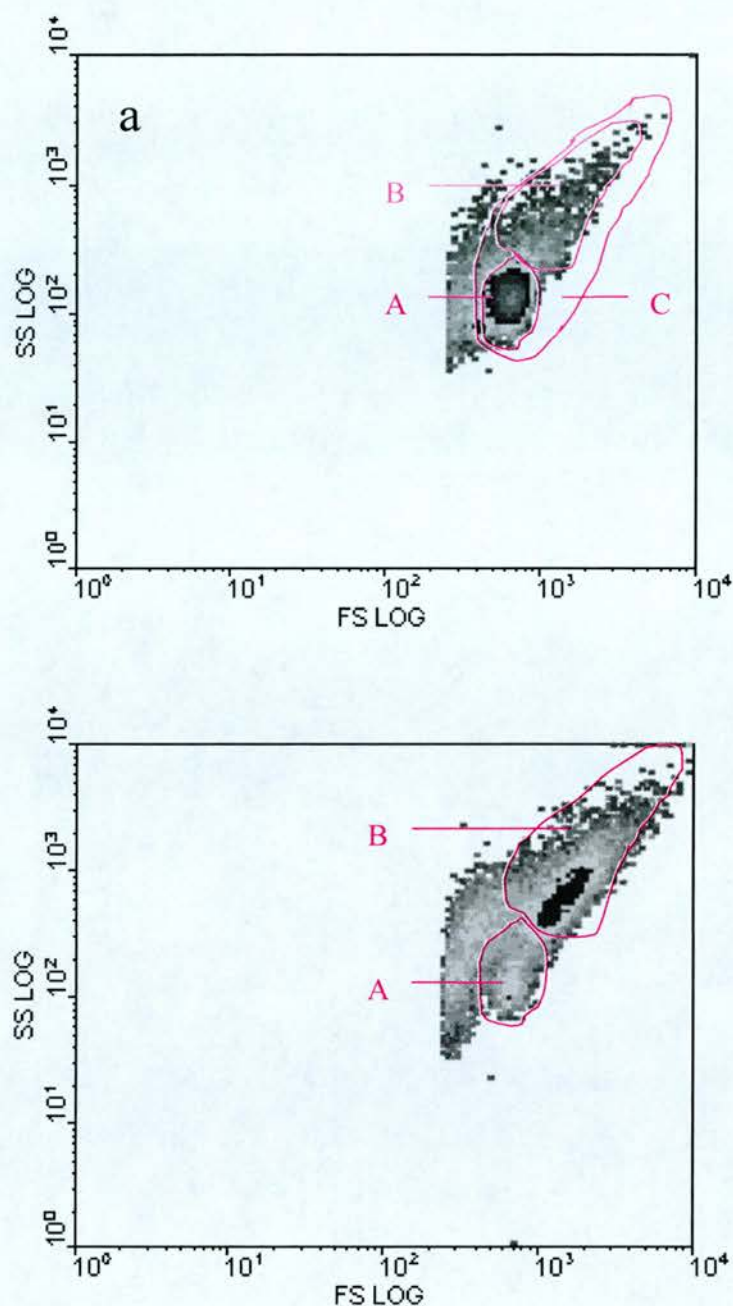


Figure 2.3. Scatter profile of mouse lymph node cells. a) the size and granularity of cells from lymph nodes draining the skin of an untreated mouse. Gates were placed around the lymphocyte region (A), the larger, more granular cells (B), and around the total cell population (C). b) profile of cells from lymph nodes draining the skin of an untreated mouse, that have been enriched for DC. Gates were placed around the lymphocyte region (A) and the larger, more granular cells (B).

2.5.8 - Annexin V staining of total and DC-enriched DLN cells

In the early stages of apoptosis, changes in the plasma membrane result in the exposure of phosphatidylserine on the outer layer of the membrane. This can be detected by flow cytometry using FITC-conjugated annexin V, which is a phospholipid binding protein with a high affinity for phosphatidylserine (Vermes *et al*, 1995). Freshly isolated or DC enriched cells (2×10^5) from lymph nodes draining the dorsal skin were incubated for 3 min at RT with FITC-conjugated annexin V (Boehringer) diluted 1/500 in annexin V binding buffer (HBSS, containing $5\mu\text{M}$ CaCl_2). Control cells were incubated in annexin V binding buffer only. Cells were then analysed immediately by flow cytometry as described in section 2.5.6.

2.6 - Detection of tumour infiltrating cells

2.6.1 - Disaggregation of tumours for flow cytometry

Mice were killed by cervical dislocation and all the tumours from one mouse were excised and pooled in 3 ml PBS containing 0.4% w/v Dispase (Dispase solution; Boehringer Mannheim, Mannheim, Germany). The tumours were cut into small fragments (approximately 2 mm^3) using a scalpel, and shaken gently in Dispase solution for 45 min at RT. The fragments were then washed twice with PBS by centrifugation, resuspended in 3 ml Dispase solution, and shaken gently for 30 min at RT (McBride, 1986). Single cell suspensions were prepared by mechanical disaggregation through a nylon cell strainer, washed twice and counted. Expression of the surface markers Thy 1.2, CD4, CD8 and CD11b was determined by flow cytometry (10^5 cells per antibody) as described in section 2.5.6.

2.6.2 - Immunoperoxidase staining of tumours

Mice were killed by cervical dislocation, whole tumours removed and snap frozen in cryomatrix (Life Sciences International, Runcorn, UK). Sections were prepared using a cryostat and allowed to dry for 1 h at RT. After fixing in ice-cold acetone for 5 min, sections were stored frozen at -20°C until use. For immunoperoxidase staining, slides were brought to RT and endogenous peroxidase blocked by incubation for 10 min in 1 % hydrogen peroxide in methanol. Slides were then washed in tap water, and blocked for 20 min at RT in 20 % (v/v) goat serum in PBS. Monoclonal rat anti-mouse antibodies (Serotec) were titrated using mouse tumour and LN sections to determine the optimal concentration; these were: CD4 (1/2000) and CD8 (1/800). The antibodies were diluted in PBS (containing 20 % v/v goat serum) and the slides incubated overnight at 4°C . Control slides were incubated in an irrelevant isotype control antibody (anti-human CD8, Serotec). After washing 3 times in PBS, slides were incubated for 30 min at RT with biotinylated goat anti-rat IgG (Serotec) diluted 1/200 (PBS, containing 20 % v/v goat serum). Slides were washed 3 times in PBS and incubated for 30 min at RT with a commercial avidin/biotinylated-HRP complex (Dako, Ely, Cambridgeshire, UK). After washing, slides were developed by the addition of diaminobenzidine (liquid DAB, Dako) for 4-7 min; slides were monitored microscopically for the appearance of staining and the reaction stopped by washing in water. Slides were counterstained with haematoxylin and mounted in DPX under a glass cover slip.

2.7 In vivo and in vitro effects of cis-UCA

2.7.1 - Topical application of UCA isomers

The UCA isomers were dissolved in dimethyl sulfoxide (10 mg/ml); this was diluted to 2 mg/ml in ethanol, and mice received 50 µl of this on shaved back skin twice a week for 3 weeks prior to tumour cell injection.

2.7.2 - Intradermal injection of cis-UCA

In one experiment, *cis*-UCA was dissolved in PBS to a concentration of 1 mg/ml; 20 µl of this was injected subcutaneously in 5 dorsal sites to give a final dose of 100µg per mouse. This was repeated twice a week for 3 weeks prior to tumour cell injection.

2.7.3 - Antibody to cis-UCA

A monoclonal antibody with specificity for *cis*-UCA (Moodycliffe *et al*, 1993) has been shown previously to abrogate some of the immunosuppressive effects of UVB, such as suppression of the DTH response to herpes simplex virus (El-Ghorr and Norval, 1995). This was diluted in PBS and 300 µl injected intraperitoneally 2 h prior to each irradiation. Control mice received an isotype control antibody (anti-Border disease virus) at the same concentration. Unirradiated controls received either *cis*-UCA antibody or control antibody.

2.7.4 - Proliferative responses to CD3 antibody

The method of Holan *et al* (1998) was used to determine the effect of *cis*-UCA on the *in vitro* proliferation of spleen cells from C3H/HeN mice in response to CD3

antibody. Spleens from normal mice were removed into ice-cold RPMI-HEPES and cell suspensions prepared by mechanical disaggregation through a nylon cell strainer. Cells were washed once, counted by trypan blue exclusion, resuspended to 3×10^6 cells/ml, and 3 ml seeded into the wells of a 6-well culture plate (Iwaki). UCA isomers were dissolved in PBS to a concentration of 2 mg/ml; these were added to the cells in varying amounts. Control cells received equal volumes of PBS. Following an incubation of 2-24 h at 37°C, cells were washed in RPMI-HEPES and resuspended to 1×10^6 cells/ml. CD3 antibody (see section 2.4.3) was added at a concentration of 2 µg/ml; 200 µl per well was seeded into a 96-well round bottomed tissue culture plate and incubated for 72 h at 37°C. ^3H -methyl thymidine was added 8h prior to the cells being harvested onto filter mats; incorporation of the radioactive thymidine was quantified as described in section 2.4.2 above.

2.7.5 - Isolation of RNA

Spleen cells were resuspended to 2×10^6 cells/ml and 1 ml seeded into the wells of 24-well tissue culture plates. The cells were incubated for 20 h in the presence of 200 µg/ml *cis* or *trans*-UCA, in the presence or absence of 2 µg/ml CD3 antibody. Cells were collected with the aid of a cell scraper (Costar, Corning, NY, USA), centrifuged for 5 min at 300 x g, and the supernatant completely removed. Total RNA was extracted using the Qiagen RNeasy mini kit according to the manufacturer's instructions. Cell pellets were resuspended in Qiagen lysis buffer; a 700 µl volume was transferred to a QIAshredder column and homogenised by centrifugation for 2 min at maximum speed. The lysate was removed and added to an equal volume of 70% ethanol. Up to 700 µl of this mixture was transferred to a spin column,

centrifuged for 15 sec and the eluate discarded. The RNA was washed by centrifugation with 700 µl Qiagen wash buffer for 15 sec and washed twice more by centrifugation with 500 µl Qiagen wash buffer for 15 sec and 2 min. The RNA was eluted by centrifugation for 1 min in 50 µl RNase-free water and stored at -70°C . Purity and concentration was determined by optical density at 260 nm and 280 nm.

2.7.6 - Reverse transcription of RNA

RNA was diluted in RNase-free water to a concentration of 2 µg in 10 µl, and denatured by heating to 65°C for 2 min. After quenching on ice, 10 µl of a reverse transcription (RT) mix was added to each sample; 1 µl RNA guard (Pharmacia Biotech), 1 µg oligo dT, 4 µl 1st strand buffer, 0.01 M dithiothreitol (Gibco, BRL), 2 units Moloney murine leukaemia virus reverse transcriptase (MMLV RT; Gibco, BRL), 1.6 µl dNTPs (25 mM mix of dATP, dCTP, dGTP, dTTP; Pharmacia Biotech). Following a 1 h incubation at 37°C , the reaction was stopped by heating to 95°C for 5 mins.

2.7.7 - Amplification of cDNA by PCR

A 2 µl volume of the RT-reaction was heated to 85°C for 5 min with 2 µl 10x PCR buffer, 3.5 mM MgCl_2 , 0.2 µl Taq DNA polymerase, 1.3 µl dNTPs (1.2 mM mix of dATP, dCTP, dGTP, dTTP) and 7.3 µl distilled water. Primers (IL-10 or β -actin, Table 2.2) were added in a 1.5 µl mix and the products amplified at 95°C for 1 min (denaturing), 65°C for 1 min (annealing) and 72°C for 1 min (polymerisation), for 40 cycles, with a final extension for 7 min at 72°C .

2.7.8 - Visualisation of PCR products

Products were run on a 1 % agarose (Gibco, BRL) gel with 10 mg/ml ethidium bromide at 40 volts, against a 100 bp DNA ladder; bands were visualised using a transilluminator (Bio-Rad).

	Primer sequence	Product size (bp)
β -actin	5' 5'-GTGGGCCGCTCTAGGCACCAA-3' 3' 5'-CTCTTTGATGTCACGCACGATTTC-3'	540
IL-10	5' 5'-ACCTGGTAGAAGTGATGCCCCAGGCA-3' 3' 5'-CTATGCAGTTGATGAAGATGTCAAA-3'	237

Table 2.2. Primer sequences used for polymerase chain reactions, and expected product size.

2.8 - Statistics

A two-tailed student's t-test was performed to determine the level of significance between experimental groups.

Chapter 3

Effect of UV irradiation with broad-band UVB, narrow-band TL01 or UVA on antigen presenting cells and contact hypersensitivity

3.1 - Introduction

Mice irradiated with FS40 sunlamps (87% of UV radiation lies between 255-340 nm, with a peak at 313 nm) for 3 weeks prior to tumour implantation show enhanced growth of tumours compared with unirradiated controls; this increased tumour outgrowth is the result of UV-induced modulation of immune responses (Donawho and Kripke, 1991).

In the present study, as will be described in Chapter 4, an attempt has been made to determine the most effective UV wavelengths for increased outgrowth of FSA tumour cells by irradiating mice with UV lamps with different spectral outputs: broad-band UVB (TL20W/12), narrow-band UVB (TL01) and UVA-I. Prior to this, it was decided to investigate the immune status of C3H/HeN mice after 3 weeks exposure to these lamps, which was the point at which they were injected with the FSA cells (see Chapter 4).

Immune unresponsiveness following UV exposure has been attributed partly to effects on epidermal antigen presenting cells. A single exposure of mice to broad-band UVB or UVA-I decreases the number of epidermal Langerhans cells (LC) (Duthie *et al*, 2000). *In vitro* exposure of LC to UVB affects the expression of co-stimulatory molecules such as CD86 and ICAM-1 (Rattis *et al*, 1998; Tang and Udey, 1992). In the current study, irradiating mice over a 3 week period with broad-

band UVB was compared with UVA to determine whether there was any correlation between changes in epidermal LC numbers and enhanced tumour outgrowth.

The decrease in epidermal LC following a single UVB exposure is accompanied by an increase in the number of dendritic cells (DC) in the lymph nodes draining the irradiated skin (Moodycliffe *et al*, 1992). UVB also causes an increase in the number of LC in the afferent lymph draining the skin of irradiated sheep (Dandie *et al*, 2001); therefore it is thought that the decrease in epidermal LC may be due to migration of the LC out of the skin to the draining lymph nodes (DLN). In the present study, the number of DC in the DLN of mice that had been irradiated for 3 weeks with broad-band UVB was assessed, to determine whether chronic UVB exposure has similar effects to a single UVB dose on antigen presenting cells. In addition, the DC were tested for expression of Ia and CD86.

The contact hypersensitivity (CH) response has often been used as an indicator of the effect of UV on cell-mediated immune responses; all 3 lamps used in this study suppress the CH response after a single exposure (El-Ghorr and Norval, 1999). The CH response is suppressed following chronic (6 weeks) exposure to broad-band UVB, but not TL01 (El-Ghorr *et al*, 1995), suggesting that there are differences in the effects of these lamps on T-cell mediated immune responses.

The suppression of CH responses following UV irradiation has been attributed partly to changes in cytokine profiles in irradiated mice. One important mediator in CH responses is IL-12; injection of anti-IL-12 antibody reduces the CH response in normal mice by 85% (Müller *et al*, 1995). IL-12 is a heterodimeric molecule composed of two covalently linked chains, p40 and p35, and is a potent inducer of Th1 responses and interferon- γ (IFN- γ) production (Manetti *et al*, 1993; Trinchieri *et*

al, 1993). Previous reports have demonstrated that exogenous IL-12 can overcome UV-induced suppression of CH responses (Schwarz *et al*, 1996), probably by reducing the DNA damage resulting from the irradiation (Schwarz *et al*, 2002). The effect of exogenous IL-12 on suppression of CH responses by the broad-band UVB protocol was examined in the current study.

LN cells from normal mice demonstrate a low level of spontaneous proliferation *in vitro*; levels of proliferation are increased in mice that have been contact sensitised (Kimber *et al*, 1986). It is not known whether UVB exposure affects the *in vitro* spontaneous proliferation of cells from LN draining the UV irradiated site. In the present study, the spontaneous and mitogen-induced proliferation of LN cells from naïve, irradiated mice was measured. The effect of broad-band UVB and exogenous IL-12 on the spontaneous proliferation of LN cells from oxazalone-challenged mice was also determined.

3.2 - Results

3.2.1 - Effect of broad-band UVB and UVA exposure on numbers of epidermal Langerhans cells

In the epidermis only LC express the ATPase enzyme on their cell surface. Mice were irradiated twice a week for 3 weeks with 1000 J/m² broad-band UVB or 10,000 J/m² UVA on the ears and shaved back. These sub-erythral doses were chosen because 3 weeks of exposure of C3H/HeN mice to these doses has previously been shown to affect epidermal LC numbers (El-Ghorr *et al*, 1995). Ears were removed 24 h after the final exposure and stained for the presence of ATPase⁺

dendritic cells, as described in section 2.3. The number of LC was significantly ($p<0.05$) reduced in both UVB and UVA irradiated epidermis (Figure 3.1).

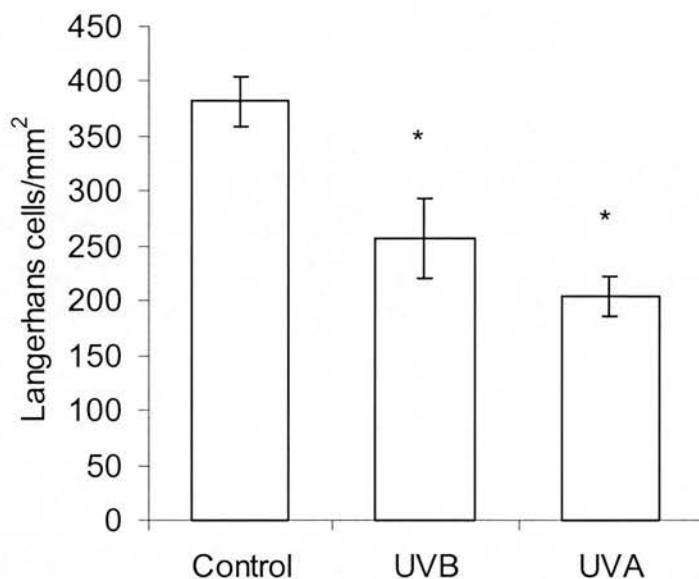


Figure 3.1. Effect of 3 week UV on epidermal LC numbers. Mice were irradiated twice a week for 3 weeks and epidermal sheets from the ears stained for ATPase⁺ LC 24 h after the final exposure. Control mice were not irradiated. Error bars show the standard error of the mean of 40 fields (SEM). * indicates a significant difference ($p<0.05$) between the control group and the irradiated groups.

3.2.2 - Effect of 3 week broad-band UVB exposure on DC in the DLN

In a study by Moodycliffe *et al* (1992), the number of DC was increased in the auricular LN following UVB irradiation of unshaved mice with a single dose, and peaked 42 h after the exposure. In the present study, mice were irradiated with a single dose of 1500 J/m^2 UVB on their shaved dorsal skin; this resulted in an increase in the number of DC in the LN draining the irradiated dorsal surface from 4575 to 6595 per LN. To determine whether the same effect was seen in mice following multiple UV exposures, mice were exposed to 1500 J/m^2 UVB twice a week for 3 weeks, and LN draining both the ears and back were pooled and enriched

for DC, as described in section 2.5.7. The results of 4 separate experiments are shown in Table 3.1. The number of DC in the LN from irradiated mice was consistently less than the number in the LN from control mice. The mean DC count for each group was calculated, and a t-test of the pooled data showed that exposure of mice to broad-band UVB for 3 weeks significantly ($p<0.05$) decreased the number of DC in the DLN.

Experiment	DC count/lymph node		
	Control	UV	% decrease
1	11101	9009	19
2	7888	4776	39
3	12295	7096	42
4	10425	7997	16
Mean	10427 (930)	7219 (903) *	29

Table 3.1. Effect of 3 week UVB irradiation on DC numbers in LN draining the irradiated site. Mice were irradiated on their shaved backs (1500 J/m^2 broad-band UVB twice a week for 3 weeks) and the number of DC in the DLN counted 42 h after the final exposure. Numbers in brackets show the standard deviation (SD). * shows a significant difference ($p<0.05$) between the control and the irradiated groups.

To assess whether chronic UV exposure affected the expression of cell surface markers involved in antigen presentation in unsensitised mice, DLN cells from unirradiated mice, and from mice that had been irradiated over a 3 week period, were enriched for DC, and the expression of Ia and CD86 determined by flow cytometry (as described in section 2.5.7). No difference was seen in the expression of either Ia (Figure 3.2a) or CD86 (Figure 3.2b) between mice exposed to multiple doses of UV and unirradiated controls. This experiment was repeated twice with similar results.

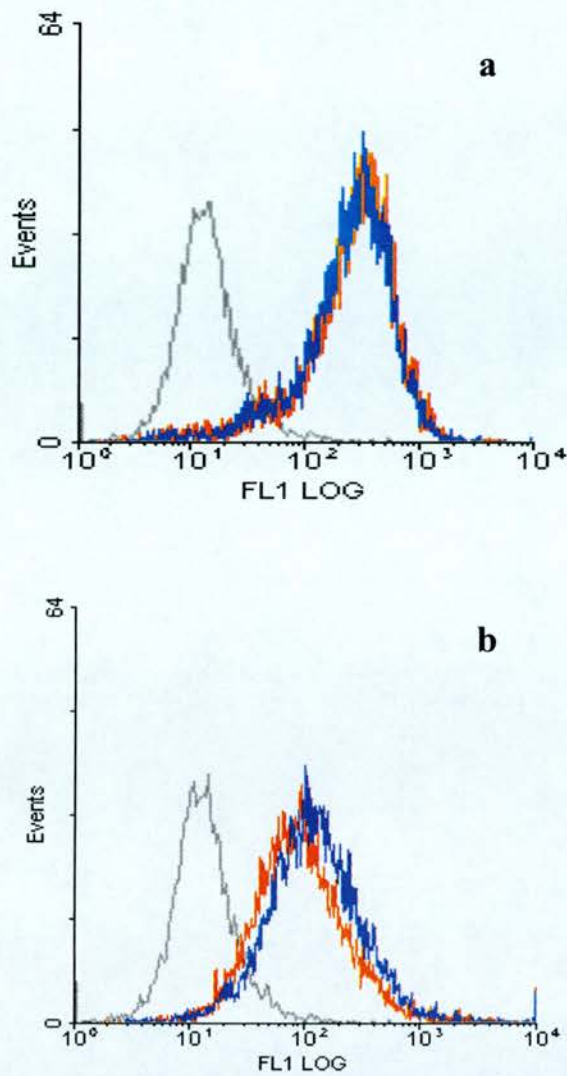


Figure 3.2. Effect of 3 week UVB exposure on DC expression of cell surface markers. Groups of 8 mice were irradiated twice a week for 3 weeks and their LN removed 42 h after the final exposure. Control mice were not irradiated. Following DC enrichment of DLN cells, the enriched populations were stained for the presence of (a) Ia and (b) CD86, and analysed by flow cytometry. The fluorescence intensity of the gated DC population is shown for control (blue line) and irradiated mice (orange line). The fluorescence intensity of DC from unirradiated mice stained with a control antibody is also shown (grey line).

To determine whether the decrease in DC number in the DLN of irradiated mice was due to increased apoptosis of these cells, DLN from unirradiated mice, and from mice that had been irradiated over a 3 week period, were enriched for DC, and the

level of annexin V binding assessed by flow cytometry (section 2.5.8). DC from control and irradiated mice demonstrated similar levels of annexin V binding, with an average (from two experiments) of 72 % positive cells for control DC and 74% for DC from irradiated mice (Figure 3.3).

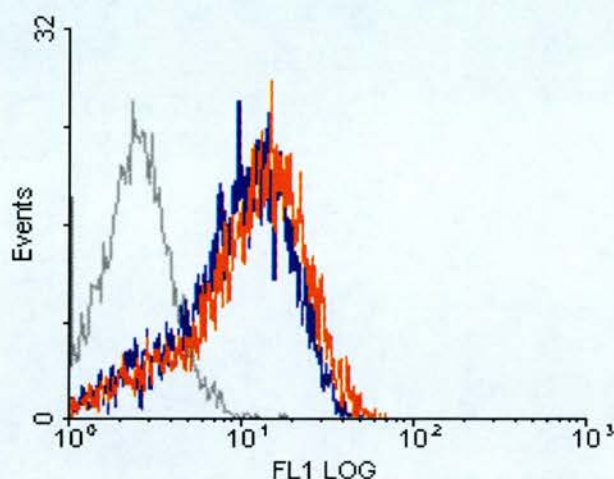


Figure 3.3. Effect of 3 week UVB exposure on annexin V binding to DC. Groups of 8 mice were irradiated twice a week for 3 weeks and their LN removed 42 h after the final exposure. Control mice were not irradiated. Following DC enrichment of DLN cells, the enriched populations were stained with FITC-conjugated annexin V, which binds to phosphatidylserine present on the surface of apoptotic cells. The fluorescence intensity of the gated DC population is shown for control (blue line) and irradiated mice (orange line). The fluorescence intensity of DC from unirradiated mice stained with a control antibody is also shown (grey line).

Additionally, the level of annexin V binding by DC in DLN cell populations that had not been enriched for DC was assessed by flow cytometry. The levels of annexin binding by DC from the total cell population were lower than the levels of binding by DC enriched populations. The average of 3 experiments was 58 % and 52 % annexin V positive cells for control DC and DC from irradiated mice respectively (not significantly different).

3.2.3 - Effect of 3 week UV exposure on the CH response to oxazalone

Mice were irradiated twice a week for 3 weeks with 1000 J/m² broad-band UVB, 10,000 J/m² TL01 or 10,000 J/m² UVA. In single exposure experiments (El-Ghorr and Norval, 1999) higher doses of these wavebands (5000 J/m², 50,000 J/m² and 500,000 J/m² respectively) were required to suppress the CH response. In the present study, sub-erythral doses were used to determine whether multiple exposures reduced the dose required for suppression of CH. Mice were sensitised with oxazalone on the irradiated back skin 3 days after the final UV irradiation (Chapter 2, section 2.2.1). All 3 UV sources significantly ($p < 0.05$) suppressed the CH response to oxazalone (Figure 3.4); the response was suppressed by 56%, 38% or 70% in mice irradiated with broad-band UVB, UVA or TL01 respectively.

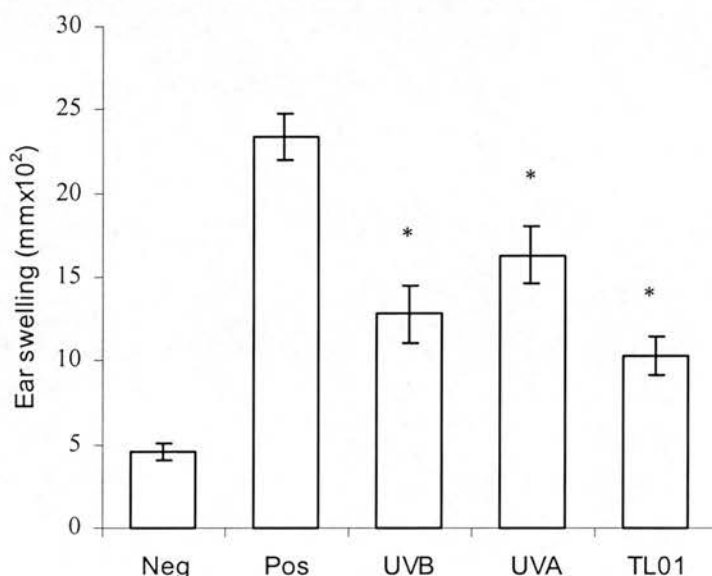


Figure 3.4. Effect of 3 week UV exposure on the contact hypersensitivity response to oxazalone. Mice (4-6 per group) were sensitised with oxazalone 3 days after the final irradiation, and challenged 8 days later. Negative controls (Neg) were not sensitised prior to challenge with oxazalone. Positive controls (Pos) were sensitised and challenged with oxazalone, but not irradiated. Error bars show the SEM. * indicates a significant difference ($p < 0.05$) between the unirradiated positive control group and the irradiated groups.

3.2.4 - IL-12 and broad-band UVB-induced suppression of the CH response

The effect of exogenous IL-12 (rIL-12) on suppression of the CH response by 3 weeks exposure to broad-band UVB was determined by irradiating mice twice a week for 3 weeks with 1500 J/m² broad-band UVB; rIL-12 was injected intraperitoneally 4 h after each irradiation (Chapter 2, section 2.1.6). Control groups received UVB only, IL-12 only, or no treatment. Mice were contact sensitised 3 days after the final irradiation as described in section 2.2.1. Once the ear swelling response after challenge had been measured, mice were re-challenged with oxazalone, and any further increase in ear swelling was measured 24 h later. This was done to assess whether a second challenge would overcome the UVB-induced suppression.

Mice receiving rIL-12 only showed no difference in their CH response compared with untreated controls (Figure 3.5a); in mice irradiated with 1500 J/m² UVB the CH response was significantly ($p<0.05$) suppressed (40% suppression) compared with the positive controls. Mice that received rIL-12 following each irradiation also had significantly ($p<0.005$) suppressed responses to oxazalone (70% suppression) compared with control mice which were either untreated or received rIL-12 only.

Following a second challenge with oxazalone, all groups showed a slight increase in ear swelling compared to that seen after a single challenge (Figure 3.5b); however, both the irradiated mice and the mice that received rIL-12 following irradiation still showed significantly ($p<0.05$) suppressed responses (22% suppression in each case).

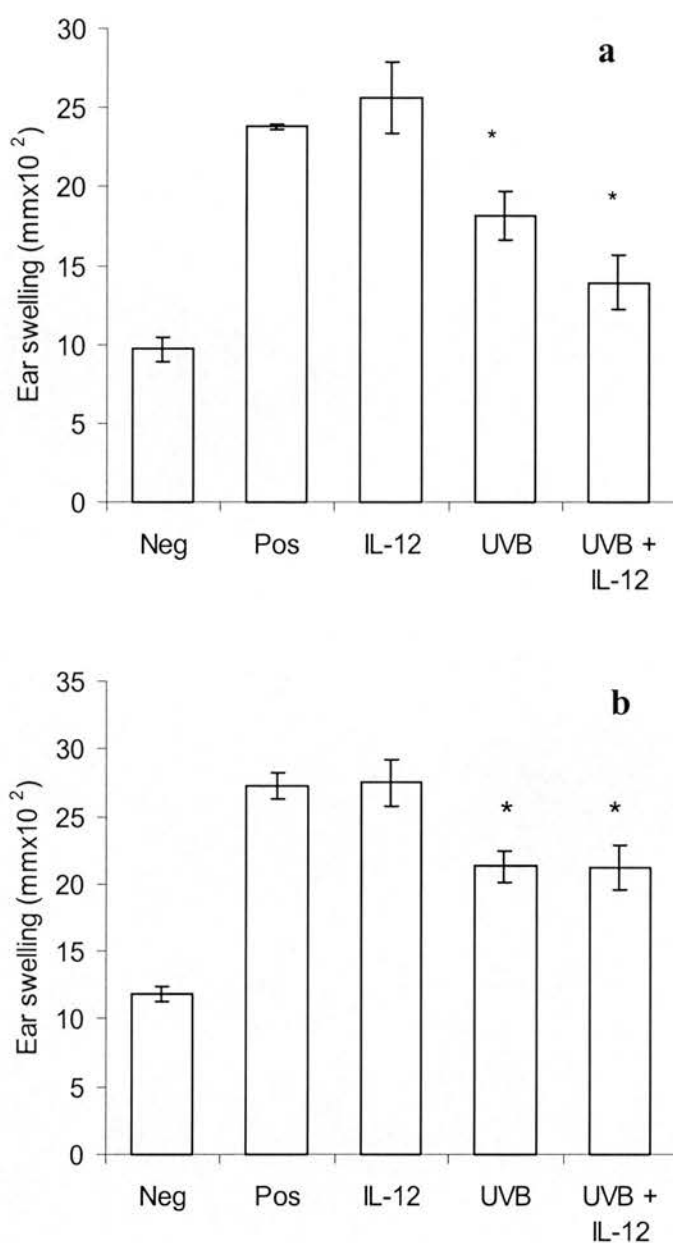


Figure 3.5. Effect of exogenous IL-12 on UVB-induced suppression of the CH response to oxazalone. Mice (6-8 per group) were irradiated for 3 weeks, and received rIL-12 4 h after each irradiation (UVB+IL-12). Control mice received either broad-band UVB only (UVB), rIL-12 only (IL-12), or no treatment prior to sensitisation (Pos). Mice were challenged 7 days after sensitisation (a) and re-challenged 24 h later (b). Negative control mice (Neg) were not irradiated and not sensitised prior to challenge with oxazalone. * indicates a significant difference between the unirradiated positive control group and the irradiated groups. Error bars show the SEM.

3.2.5 - Effect of UVB exposure on the *in vitro* proliferation of LN cells from naïve and challenged mice

To assess whether UV irradiation had any effect on the spontaneous proliferation of DLN cells from unchallenged mice, mice were irradiated twice a week for 3 weeks with broad-band UVB and their LN removed 24 h after the final irradiation (sections 2.5.1 and 2.5.2). No difference was seen in spontaneous proliferation of cells from UV-irradiated mice compared with unirradiated controls (Figure 3.6). This experiment was repeated 3 times with similar results.

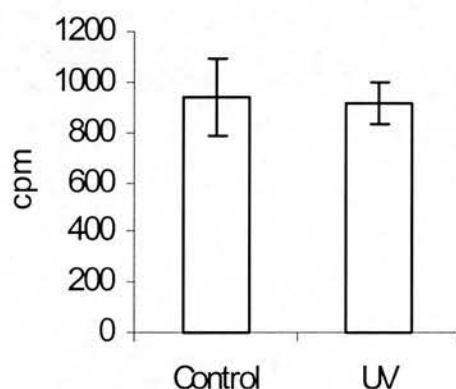
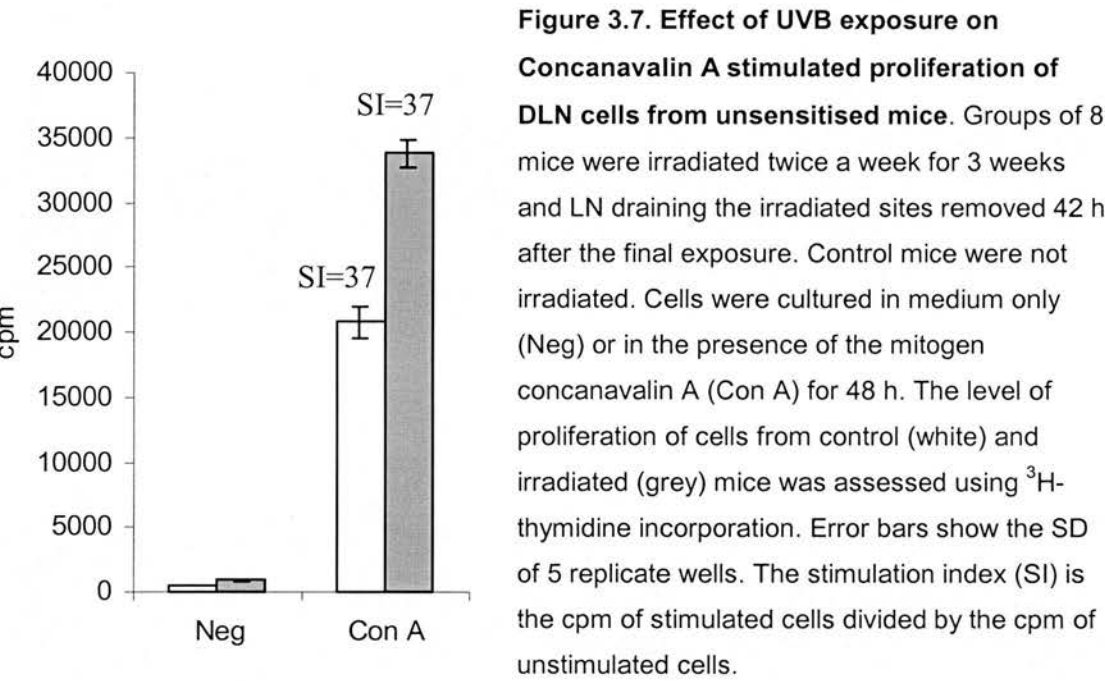


Figure 3.6. Effect of UVB exposure on proliferation of DLN cells from unsensitised mice. Groups of 8 mice were irradiated twice a week for 3 weeks and LN draining the irradiated sites removed 42 h after the final exposure. Control mice were not irradiated. Cells were cultured for 24 h and the level of proliferation was assessed using ^3H -thymidine incorporation. The average cpm for 3 separate experiments is shown. Error bars show the standard deviation (SD).

In one experiment, proliferation of LN cells stimulated with the mitogen concanavalin A ($2.5 \mu\text{g/ml}$ for 48 h) was measured, as described in section 2.5.3. Figure 3.7 demonstrates that the mitogen-stimulated proliferation of LN cells from mice irradiated with UVB for 3 weeks was increased compared to controls, however

there was no difference in the stimulation index between control and UV-irradiated groups.



The *in vitro* spontaneous proliferation of DLN cells (Chapter 2, section 2.5.2) from oxazalone-sensitised mice that had been challenged twice with oxazalone is shown in Figure 3.8. The unsensitised mice receiving 2 challenges with oxazalone showed significantly ($p<0.05$) higher spontaneous proliferation compared with all of the sensitised groups. The spontaneous proliferation of DLN cells from rIL-12 treated mice (irradiated or unirradiated) was significantly higher ($p<0.05$) than that of the unirradiated positive controls. There was no difference in spontaneous proliferation of DLN cells from irradiated mice compared with unirradiated positive controls.

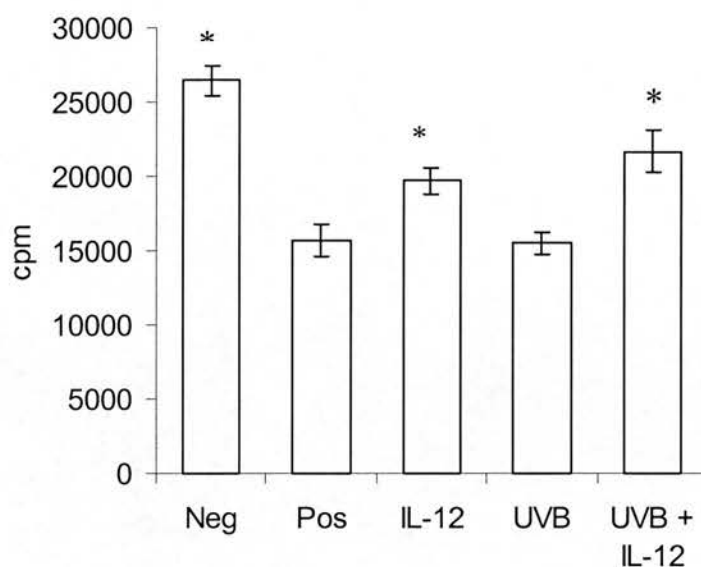


Figure 3.8. Effect of UVB and exogenous IL-12 on spontaneous proliferation of DLN cells from oxazolone-challenged mice. Mice were treated as described in Figure 3.4. Lymph nodes draining the challenge site were taken 24 h after the second challenge and cultured overnight in the presence of ^3H -thymidine; the average of 5 replicate wells is shown. Error bars show the SD. * indicates a significant difference ($p < 0.05$) between the negative or rIL-12 treated groups and the positive control group.

3.3 - Discussion

3.3.1 - Effect of broad-band UVB and UVA on numbers of epidermal LC

Exposure of mice for twice a week for 3 weeks to sub-erythemal doses of broad-band UVB (1000 J/m^2) and UVA ($10,000 \text{ J/m}^2$) resulted in a decrease in the number of LC in the UV-irradiated site. This is in agreement with previous studies where epidermal LC numbers were reduced following multiple exposures of mice to sub-erythemal doses of broad-band UVB (500 J/m^2 , El-Ghorr *et al*, 1995) or UVA (19320 to 42446 J/m^2 , Bestak and Halliday, 1996). In the study by El-Ghorr *et al*, irradiation of mice 3 times a week for 3 weeks with 1000 J/m^2 UVB (TL20/12) resulted in a decrease in epidermal LC numbers at a greater magnitude (70%) than seen here (30%), probably relating to the higher number of weekly doses used. That

study also showed a decrease in LC numbers following 3000 J/m² TL01 3 times a week for 3 weeks; determination of LC numbers following TL01 exposure in this system would have made the data more complete.

The decrease in LC numbers (~50%) following a 3 week exposure to UVA was unexpected because in a previous study, although a single MED of broad-band UVB (5000 J/m² in that study) was sufficient to deplete epidermal LC numbers, no decrease was seen following a single MED of UVA-I (500,000 J/m²) (Duthie *et al*, 2000). The use of a chronic low-dose UVA regimen may be responsible for the differential effects on LC numbers. Contaminating UVB wavelengths (0.5% of total emission of UVA lamp) were not removed with Cation X in the current study, such that mice received a very low dose of UVB (50 J/m² UVB per 10,000 J/m² UVA). The decrease in epidermal LC following UVA irradiation may have been due to the UVB wavelengths; however, in single exposure experiments, at least 2500 J/m² broad-band UVB (TL20W/12) was required to cause significant depletion of epidermal LC (Duthie *et al*, 2000). In the present study, UVA irradiated mice received a total dose of only 300 J/m² UVB; however it could be that multiple exposures to very small amounts of UVB reduces the total dose required to affect epidermal LC numbers. In determining an action spectrum for UV-induced LC depletion, Obata and Tagami (1985) demonstrated that a single exposure to wavelengths of 320 nm and below reduced LC numbers, while wavelengths of 340 nm and above (UVA-I) had no effect. To rule out the possible contribution of the contaminating UVB wavelengths to LC loss following UVA irradiation, this experiment could be repeated using the Cation X filter such that mice are exposed only to UVA-I.

3.3.2 - Effect of 3 week broad-band UVB on DC in DLN

A number of studies have provided evidence that the decrease in LC numbers following UV exposure is due to the migration of LC from the skin. Experiments involving cannulation of draining afferent lymph vessels have demonstrated an increase in lymph flow and cell output after UVB-exposure of human volunteers (Yawalkar *et al*, 1998), and an increase in the number of LC in afferent lymph draining the skin of UVB-irradiated sheep (Dandie *et al*, 2001).

In the current study, as reported previously by Moodycliffe *et al* (1992), the number of DC in the DLN was increased in mice that had received a single dose of UVB, providing evidence that UVB exposure results in migration of epidermal LC to the DLN; in a previous study, a single UVA-I exposure also increased DC number in the DLN (Duthie *et al*, 2000). In contrast, following a chronic 3 week UVB protocol, the number of DC in DLN was decreased compared to unirradiated mice. In the study by Moodycliffe *et al* (1992), the increase in DC number in the DLN of irradiated mice reached a maximum at 42 h after the exposure, and then decreased rapidly to the levels found in unirradiated mice by around 60 h. This decrease may be due to the re-circulation of the DC; alternatively, the DC that have migrated from the UVB-exposed skin may apoptose upon reaching the LN. In the present study, an attempt was made to assess the level of apoptosis of DC from the DLN of irradiated mice using annexin V, which binds to phosphatidylserine, and early marker on apoptotic cells. No difference in the level of apoptosis in DC in DLN cell populations from unirradiated and irradiated mice was indicated; however a high level of binding was seen in DLN cells that had been enriched for DC. Since annexin V binding is an early

marker of apoptosis, it is likely that the *in vitro* manipulation of the cells resulted in a large percentage of early apoptotic cells. Using a marker for cells in the later stages of apoptosis and taking LN from mice at various stages during the chronic irradiation protocol might have yielded more information on the levels of apoptosis of DC in the DLN of UV-irradiated mice.

In vitro studies have demonstrated that UVB-irradiated LC only show increased apoptosis following at least 2 days in culture (Rattis *et al*, 1998; Tang and Udey, 1992), leading to the suggestion that LC migrating from the skin apoptose once they have reached the DLN. Similarly, following skin painting with an irritant, DC accumulate in the DLN, but disappear rapidly after 2 days (Ruedl *et al*, 2000), an effect attributed to the death of the DC, since no DC have been observed leaving LN via the efferent lymphatics (Smith *et al*, 1970). This is in contrast to DC migrating from the skin in unsensitised mice, which demonstrate a turnover of around 30 days (Ruedl *et al*, 2000). Thus, while DC that migrate from the skin in normal, untreated mice can remain in the DLN for some time, LC that have migrated from UVB-irradiated skin, some of which demonstrate DNA damage (Sontag *et al*, 1995; Kölgen *et al*, 2002), are perhaps more likely to undergo apoptosis, decreasing the overall DC numbers in the DLN of chronically irradiated mice.

Despite the decrease in DC numbers in the DLN of irradiated mice, no difference in expression of the cell surface molecules Ia and CD86 was found on DC from DLN of UV-exposed mice, compared to DC from unirradiated mice. Therefore, the DC that remain in the DLN following multiple UV exposures express normal levels of at least two of the molecules involved in antigen presentation to, and stimulation of, T cells. The decrease in both epidermal LC and DC in the DLN

suggests that there is a lack of available antigen presenting cells in chronically UVB-irradiated mice, which could either lead to less effective initiation of an immune response, or make antigen presentation by other cells such as macrophages more important in these mice.

3.3.3 - Effect of 3 week UV irradiation on the CH response

Exposure of mice twice a week for 3 weeks to broad-band UVB (1000 J/m²), narrow-band TL01 (10,000 J/m²) or UVA (10,000 J/m²) significantly suppressed the CH response to oxazalone. In the chronic exposure study by El-Ghorr *et al* (1995) a six-week exposure to 3000 J/m² TL01, 3 times a week, failed to suppress the CH response; the higher dose of TL01 used in the present study could account for the suppression. A single dose of 5000 J/m² UVB, 50,000 J/m² TL01 or 500,000 J/m² UVA-I is the minimum dose required to suppress the CH response in C3H/HeN mice (El-Ghorr and Norval, 1999); use of multiple exposures in this study has reduced the dose required for suppression.

Exposure to broad-band UVB or UVA over a 3 week period decreased the number of epidermal LC, and suppressed the CH response to oxazalone, suggesting that UV effects on LC may play a role in UV-induced suppression of CH. A correlation between epidermal LC density and the induction of CH responses has been shown (Toews *et al*, 1990); thus, a decrease in LC numbers by UV exposure may be enough to suppress CH. In a human study however, susceptibility to UVB-induced suppression of CH was not related to the number of epidermal LC (Skov *et al*, 1998).

Alcalay and Kripke (1990) demonstrated that DLN cells taken from mice sensitised through UV-irradiated skin had reduced ability to initiate a CH response in normal mice as compared with DLN cells taken from unirradiated, sensitised mice. It is therefore possible that UV-induced immune suppression could result from altered antigen presentation following irradiation by UV-damaged LC. The loss of epidermal LC following UV-irradiation is accompanied by the infiltration of inflammatory cells such as macrophages and neutrophils (Cooper *et al*, 1993). Hammerberg *et al* (1996) showed that UV-induced tolerance and suppression of CH could be reversed by *in vivo* anti-CD11b treatment; reversal of tolerance was associated with reduced numbers of infiltrating monocyte/macrophage cells in the irradiated site. Using DLN cells from fluorescein isothiocyanate (FITC)-sensitised mice, it has been shown that DLN cells from irradiated mice induce significantly less proliferation of FITC-specific T cells *in vitro*; of the Ia⁺ dendritic cells, a significantly higher percentage of the cells from UV-irradiated mice expressed macrophage markers, and fewer contained the LC-specific Birbeck granules (Bucana *et al*, 1994). It is possible that antigen presentation by cells other than LC following UV-irradiation may result in a tolerogenic response.

The use of multiple doses of UV in this study is perhaps more reflective of the everyday sunlight exposure received by individuals; however the effects of UV on murine LC may not equate with the response of human epidermal cells. Following *in situ* UVB exposure with doses that alter the morphology and stimulatory capacity of mouse LC, no effect is seen on the morphology or stimulatory capacity of human epidermal cells (Goettisch *et al*, 1998). Kremer *et al* (1997) showed that LC from

UVB-irradiated human epidermal sheets retained their ability to stimulate the proliferation of allogeneic T cells, despite having UV-induced DNA damage.

3.3.4 - IL-12 and UV-induced suppression of CH

Mice irradiated for 3 weeks with 1500 J/m² broad-band UVB showed significantly suppressed CH responses; mice injected with rIL-12 after each irradiation had even more suppressed CH responses. There was no difference in the CH response of unirradiated mice receiving or not receiving rIL-12.

The suppression of CH responses following UV irradiation has been attributed partly to changes in cytokine profiles in irradiated mice. Irradiation with UVB results in increased epidermal IL-10 expression, whereas UVA irradiation increases the levels of epidermal IL-12 and IFN- γ (Shen *et al*, 1999). In unirradiated mice IFN- γ , IL-12 and IL-4 are detectable in spleen and DLN after sensitisation with oxazalone (Garssen *et al*, 1999); UV exposure daily for 4 days prior to sensitisation decreases the levels of both IFN- γ and IL-12. Schwarz *et al* (1996) showed that injection of rIL-12 prior to sensitisation abrogated the suppression of CH in mice that had received 4 consecutive daily exposures to UV. Transfer of spleen and DLN cells from UV-irradiated, sensitised mice to naïve mice suppressed the CH response when these mice were challenged; no suppression was seen in mice that received lymphoid cells from UV-irradiated, rIL-12 treated donors. Similarly, rIL-12 abrogated the suppression of the DTH response to allogeneic spleen cells following a single dose (15,000 J/m²) of UVB (Schmitt *et al*, 1995). Schwarz *et al* (2002) showed that rIL-12 treatment repaired UVB-induced DNA damage; it is suggested that this prevents the production of IL-10, which is thought to be released following DNA damage

(Nishigori *et al*, 1996), and which has been shown to be important in UV-induced immunosuppression (Beissert *et al*, 1996; Kang *et al*, 1994).

The suppression of the CH response despite injection of rIL-12 following UV irradiation in this study was therefore unexpected. In the study by Schwarz *et al* (1996) mice were exposed to 1000 J/m² UV (FS-20 sunlamps) for 4 consecutive days and mice were sensitised with DNFB one day after the final irradiation; rIL-12 was injected 3 h prior to sensitisation. In a similar study (Müller *et al*, 1995), mice received 5 consecutive daily doses of UV (100 J/m²); injection of 500 ng rIL-12 on the day of the final UV-exposure abrogated UV-induced suppression of CH to DNFB. In the present study, rIL-12 was injected 4 h after each irradiation, and the mice sensitised 3 days after the final UV exposure; the difference in timing of rIL-12 injection may account for the lack of an effect of rIL-12 on UV-induced suppression of CH.

It is possible that the amount of rIL-12 injected was insufficient to overcome the suppression induced by 3 weeks exposure to 1500 J/m² broad-band UVB. In the study by Schwarz *et al*, mice received 100 ng rIL-12 3 h prior to sensitisation; while this was sufficient to overcome suppression induced by 4 exposures of 1000 J/m² UVB, injection of 50 ng rIL-12 was not. In the present study, mice received 50 ng of rIL-12; this dose was chosen because it was injected after each irradiation. Higher doses of IL-12 might have been effective in reversing the UV-induced suppression; however, repeated injection of rIL-12 can have adverse effects such as weight loss and splenomegaly (Ryffel, 1997). Additionally, daily injection of rIL-12 (500 ng per mouse) has been shown to cause suppression of a DTH response to alloantigen (Koblish *et al*, 1998). It is thought that immunosuppression due to high doses of IL-

12 is caused by nitric oxide-induced T cell apoptosis (Lasarte *et al*, 1999). Repeated injection of 50 ng rIL-12 had no effect on the CH response in unirradiated mice, although suppression of CH in irradiated mice (40%) was higher (but not significantly) in those treated concomitantly with rIL-12 (70%). It is possible that in this protocol rIL-12 is enhancing UV-induced suppression through the action of nitric oxide; determination of apoptosis levels in the spleen or DLN, or treatment of mice with a nitric oxide synthase inhibitor, would perhaps have shown if this had a role in the increased suppression seen in rIL-12 treated UV-irradiated mice.

Following a second challenge with oxazalone, the amount of UV-induced suppression was decreased from 40% to 22%; in single challenge experiments the amount of UV-induced suppression decreases as the concentration of the challenge contact sensitizer is increased (Miyauchi and Horio, 1995). Previous studies by Shimizu and Streilein (1994a, 1994b) demonstrated UVB-induced tolerance, such that upon a second challenge with a contact sensitizer (14 days after the first challenge), the CH response in irradiated mice remained significantly suppressed compared with controls. It is possible that challenging the mice a second time in the present study is causing the UVB-induced tolerance to be overcome; it would have been interesting to see if further challenges would have further decreased the suppression in UV-irradiated mice.

3.3.5. - Effect of UVB exposure on *in vitro* proliferation of DLN cells

Despite the decrease in DC number in the DLN of mice exposed to broad-band UVB over a 3 week period, there was no change in the *in vitro* spontaneous proliferation of the DLN cells. In a previous study, mice exposed to a single UVB

dose (1440 J/m^2) showed an increased proliferative response to Con A (El-Ghorr and Norval, 1997); however, in the present study, the ability of DLN cells to proliferate in response to Con A was not affected by 3 weeks exposure to UVB. These results are in agreement with a study using human volunteers, who received whole body irradiation with 0.8 MED UVB on 5 consecutive days. The irradiation had no effect on the *in vitro* spontaneous or Con A stimulated proliferation of peripheral blood leukocytes (Weichenthal *et al*, 2000).

In mice that had been challenged with oxazalone, the DLN cells from all groups showed a very high spontaneous proliferation, probably due to the mice being challenged twice. DLN cells from mice that had been previously sensitised with oxazalone showed a lower spontaneous proliferation compared with unsensitised mice. This is perhaps unexpected, however it is not known whether DLN cells from mice that have been sensitised and are then challenged a single time show an increased spontaneous proliferation compared with mice that have not been previously sensitised. Here a second challenge with oxazalone resulted in a higher proliferation in unsensitised mice compared with previously sensitised mice. It is possible that the proliferation of LN cells from previously sensitised mice had reached a peak on or before the second challenge.

Proliferation of DLN cells from mice that had received rIL-12 (irradiated and unirradiated) was increased compared with controls, which is consistent with the role of IL-12 as a mediator of CH responses. As with LN cells removed from naïve mice and stimulated *in vitro*, UVB exposure had no effect on proliferation of cells from mice that had received a challenge *in vivo*. Challenge of the mice a single time might have given more informative results, as it would have shown whether the increase in

proliferation that is seen in mice that have been previously sensitised is affected by prior exposure to UVB.

3.4 - Summary of Chapter 3

Following 3 weeks of broad-band or narrow-band UVB, or UVA exposure, irradiated mice have decreased numbers of epidermal LC, and suppressed CH responses; thus the immune systems of irradiated mice at the time of fibrosarcoma cell injection are altered compared with unirradiated controls. Mice irradiated with a single dose of UVB have increased DC numbers in the LN draining the irradiated skin, which is thought to be due to LC migration from the skin. Mice exposed to UVB six times over a period of 3 weeks have decreased DC numbers in the skin draining LN compared with control, unirradiated mice. This decrease suggests that there is a lack of available APC in chronically UVB irradiated mice, which may play a part in UVB-induced immunosuppression.

Injection of rIL-12 after a single UV exposure has been shown previously to reverse UV-induced suppression, suggesting that induction of suppressor cells by UV exposure is overcome by rIL-12 treatment. This study has demonstrated that in a chronic protocol, rIL-12 did not reverse UVB-induced suppression, rather, the suppression was greater in irradiated mice treated with rIL-12. Perhaps the use of low doses of rIL-12 in a chronic UVB protocol increases UV-induced suppressor cell activity, rather than abrogating it.

Exposure of mice to UVB over a period of three weeks has no effect on the *in vitro* spontaneous proliferation of DLN cells from either unsensitised mice, or mice that have been challenged twice with oxazalone. UVB exposure also had no effect on

the *in vitro* proliferation of DLN cells in response to mitogenic stimulation; thus, the immunosuppression resulting from chronic UVB exposure is not related to an effect on the proliferative ability of cells from LN draining the irradiated site.

Chapter 4

Effect of UV irradiation with broad-band UVB, TL01 or UVA-I on outgrowth of fibrosarcoma cells *in vivo*

4.1 - Introduction

Kripke and Fisher (1976) demonstrated that antigenic UV-induced skin tumours, which are rejected upon transplantation into immunocompetent mice, grow progressively when transplanted into mice that have been UV-irradiated for several weeks prior to tumour implantation. Experiments using spleen cell transfers revealed that the effect of UV exposure was immunological. Since then, it has been shown that UV radiation has numerous effects on the immune system, including alterations of epidermal antigen presenting cell numbers (Duthie *et al*, 2000), changes in cytokine expression (Shen *et al*, 1999), and suppression of CH and DTH responses (El-Ghorr and Norval, 1999).

A study using Skh-1 albino hairless mice demonstrated that the most effective wavelength for induction of skin cancer was 293 nm, which lies within the UVB (290-320 nm) range (de Gruijl *et al*, 1993). A shoulder of effectiveness at 10^{-4} of the maximum resulted from exposure of mice to wavelengths over 340 nm, suggesting a possible role for UVA (320-400 nm) in the generation of skin cancer.

It is not known whether there is a most effective wavelength for the suppression of anti-tumour immune responses. In the study by Kripke and Fisher (1976), the lamps used emitted broad-band UV wavelengths. Using a UV-induced fibrosarcoma, De Fabo and Kripke (1980) demonstrated that mice exposed to an FS40 sunlamp (87% of total output lies between 255-340 nm) were rendered susceptible to enhanced outgrowth of

subsequently implanted tumours. Increased tumour outgrowth in irradiated mice was not affected by the removal of wavelengths below 275 nm; however, removal of wavelengths below 315 nm abrogated the enhanced outgrowth of tumours in irradiated mice. Chapter 2 and previous studies have demonstrated that both TL01 (311-313 nm) and UVA (340 – 400 nm) exposure affects immune parameters in mice, including the suppression of the CH response, and loss of LC from the epidermis. The immune alteration that leads to increased tumour outgrowth in irradiated mice can be transferred by spleen cells to unirradiated mice, suggesting that the UV-induced suppression of anti-tumour immunity is systemic. Additionally Fisher and Kripke (1977) demonstrated that when two mice, one irradiated and one unirradiated, were joined parabiotically, subsequently implanted tumours grew progressively in both mice.

In the present study, using a moderately immunogenic fibrosarcoma (FSA) cell line, the effect of exposure of mice to the UVA and TL01 lamps on the growth of subsequently injected FSA cells was compared with that of a lamp emitting broad-band UVB (TL12, 280-320 nm). The systemic effects of broad-band UVB exposure on enhanced tumour cell outgrowth in irradiated mice were assessed by injection of FSA cells either into a non-irradiated skin site, or intravenously, which results in the formation of measurable colonies in the lung (Milas *et al*, 1974).

Previous studies have demonstrated that injection of exogenous IL-12 between UV exposure and antigen application can overcome UV-induced suppression of CH and DTH responses; IL-12 injection can also break UV-induced tolerance (Schwarz *et al*, 1996; Schmitt *et al*, 1995). As will be discussed in more detail in chapter 6, *in vitro* incubation of tumour antigen pulsed epidermal cells with *cis*-UCA suppresses their ability to induce and elicit anti-tumour responses upon injection into mice (Beissert *et*

al, 1997); this suppression can be abrogated by incubation of the epidermal cells with exogenous IL-12 (Beissert *et al*, 2001). In the present study, the effect of *in vivo* administration of exogenous IL-12 on UVB-enhanced tumour outgrowth is examined.

4.2 - Results

4.2.1 - Growth of FSA cells after injection into the dorsal skin of C3H mice

In order to determine a suitable number of FSA cells to use when testing the effects of UV on FSA cell outgrowth, untreated mice were injected in four shaved dorsal sites with varying numbers of FSA cells, as described in section 2.4.1. Figure 4.1 shows that following injection of 10^3 FSA cell in four dorsal sites, no palpable tumours were apparent by 14 days post inoculation. Injection of 10^4 FSA cells resulted in the appearance of palpable tumours at seven days, and by day 14 tumours were apparent in around 30% of the inoculation sites; injection of 10^5 cells gave rise to tumours in all the inoculation sites. It was therefore decided to use 7.5×10^4 FSA cells when assessing the effect of UV on tumour cell outgrowth, as injection with too few, or too many, FSA cells can give rise to tolerance and the induction of suppressor cells (McBride and Howie, 1986).

Figure 4.2 shows a mouse 10 days after injection of 7.5×10^4 FSA cells in 4 dorsal sites, where tumours were first apparent by day 4 post-inoculation. The majority of tumours grew to approximately 4 mm diameter, at which point the growth rate declined (around day 15). Some tumours continued to grow until they reached 10 mm diameter, at which point the mice were killed for humane reasons; mice were also killed when tumours (usually the largest) showed signs of ulceration (around day 24).

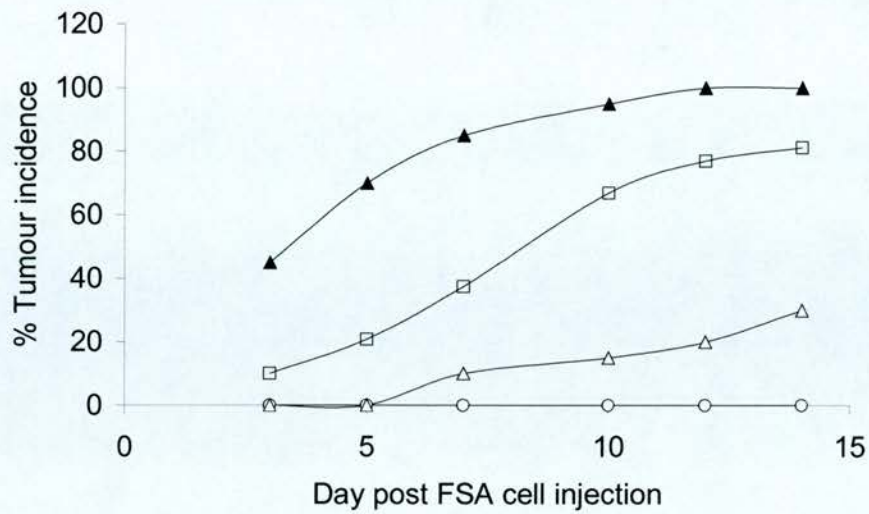


Figure 4.1. Incidence of tumours following injection of varying numbers of FSA cells. Mice (4–6 per group) received four injections into shaved dorsal skin of 10^3 (O), 10^4 (Δ), 8.4×10^4 (\square) or 10^5 (\blacktriangle) FSA cells per site. Tumour incidence is the percentage of FSA injected sites where a palpable tumour formed.

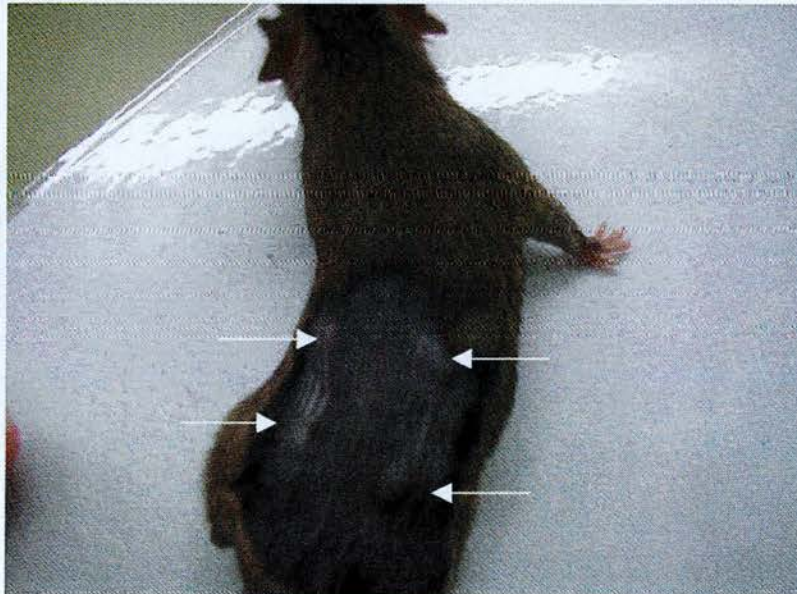


Figure 4.2. Growth of tumours following subcutaneous injection of FSA cells. Injection of 7.5×10^4 FSA cells in four dorsal sites gave rise to four visible tumours in this mouse, indicated by the arrows, 10 days later.

4.2.2 - Effect of broad-band UVB exposure on outgrowth of FSA cells

A dose of 1500 J/m^2 broad-band UVB (TL12) represents one MED in C3H mice; previous studies that demonstrate an increase in tumour growth following UVB exposure have used doses much higher than 1 MED (De Fabo and Kripke, 1980; Kripke and Fisher, 1976). Exposure of mice twice a week for three weeks to 1000 J/m^2 UVB results in suppression of the CH response (section 3.2.3); therefore this dose was initially used to determine whether prior exposure of mice to sub-erythral doses of UVB caused increased tumour outgrowth. Mice were irradiated on their shaved dorsal skin twice a week for three weeks; 7.5×10^4 FSA cells were injected 24 h after the final irradiation (section 2.4.1). The size of the resulting tumours was no different to that of the tumours in unirradiated controls (Figure 4.3). Increasing the dose of UVB to 1200 J/m^2 however, resulted in a significant ($p < 0.05$) increase in the size of the tumours (Figure 4.3). Following FSA cell injection, one group of mice continued to receive 1200 J/m^2 twice a week; continuation of the UVB exposure did not further increase the UV-enhanced outgrowth of the tumours (Figure 4.3).

To determine whether the three week period of UVB exposure was necessary to enhance FSA cell outgrowth, mice were exposed to 1500 J/m^2 UVB twice a week for one, two or three weeks and 7.5×10^4 FSA cells injected 24 h after the final irradiation. In figure 4.4a, mice exposed to UVB twice a week for three weeks showed significantly ($p < 0.05$ compared to unirradiated controls) increased tumour outgrowth from day 13, the first day of measurement.

Exposure of mice to UVB twice a week for only two weeks did not have a consistent effect on tumour cell outgrowth. In the experiment represented by Figure 4.4a, mice irradiated for two weeks prior to FSA cell injection showed increased tumour

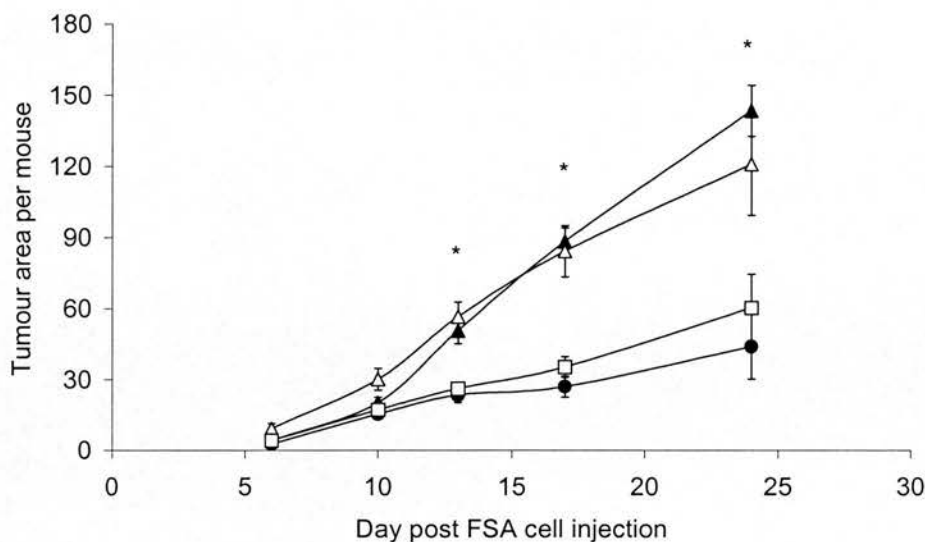


Figure 4.3. Growth of FSA cells in C3H mice following 3 weeks broad-band UVB (TL12) exposure. Groups of mice (15-17) were irradiated twice a week for 3 weeks with 1000 (□) or 1200 (△) J/m² TL-12. Control mice were not irradiated (●). FSA cells were injected 24 h after the final UV exposure. One group continued to receive 1200 J/m² TL12 twice a week following FSA cell injection (▲). * indicates a significant difference ($p < 0.05$) between the unirradiated group and the groups irradiated with 1200 J/m². Error bars show the SEM.

outgrowth compared to unirradiated controls, although this was to a lesser extent than mice exposed to UVB for three weeks, and was not significantly more than unirradiated controls. Whereas two weeks UVB exposure had no significant effect on tumour outgrowth in Figure 4.4a, Figure 4.4b shows that two weeks exposure of mice to 1500 J/m² UVB prior to FSA cell injection resulted in a significant ($p < 0.05$ on days 7 to 17) decrease in the size of the resulting tumours compared with unirradiated controls.

Exposure of mice to UVB for only one week had no effect on the outgrowth of subsequently implanted FSA cells, as compared to unirradiated controls (Figure 4.4b). It was therefore decided that the three week protocol was the most suitable for experiments using the broad-band UVB source.

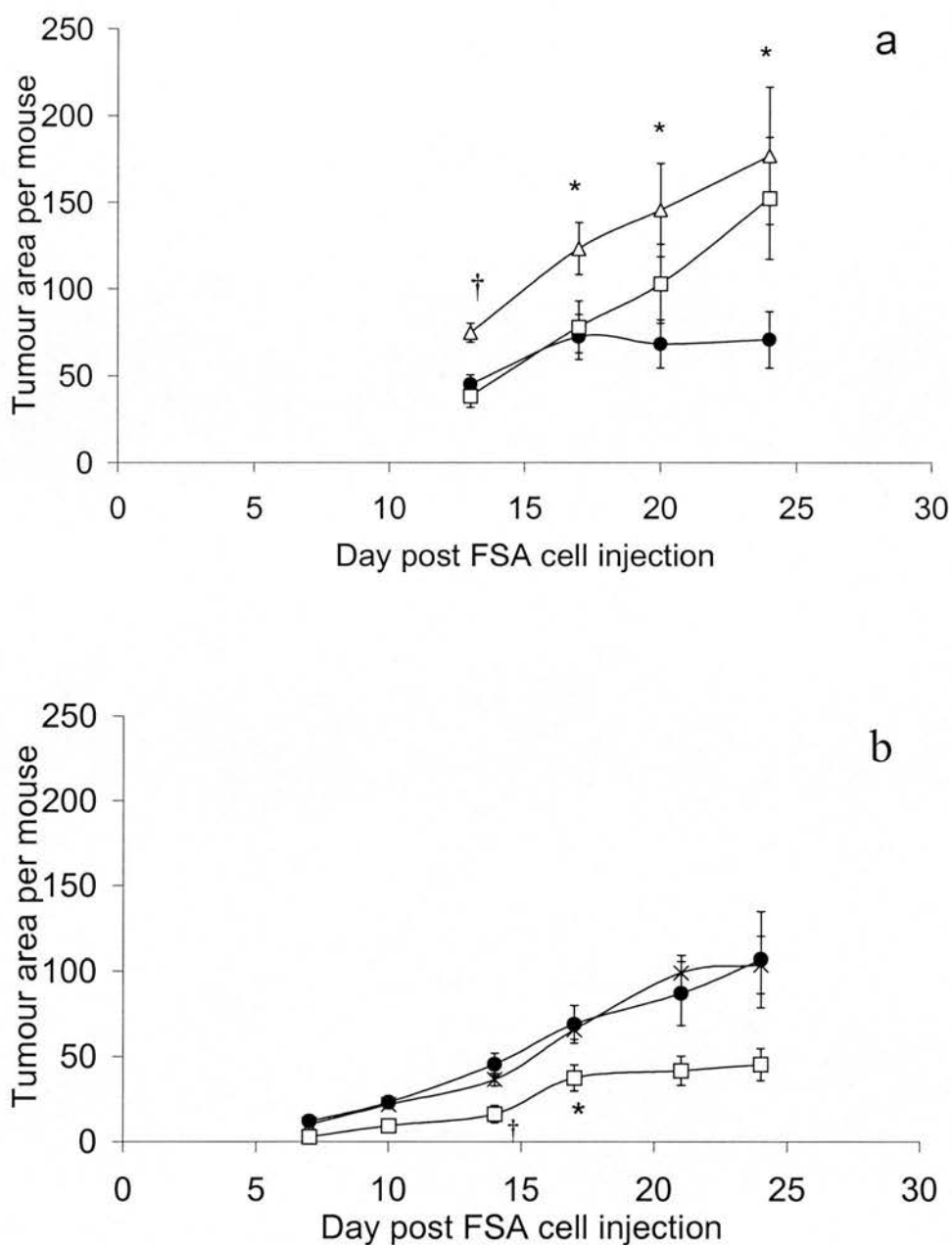


Figure 4.4. Growth of FSA cells following 1,2 or 3 weeks broad-band UVB (TL-12) exposure. Groups of mice (8-9) were irradiated with 1500 J/m² broad-band UVB twice a week a) for 2 (□) or 3 (△) weeks prior to FSA cell injection or b) for 1 (x) or 2 (□) weeks prior to FSA cell injection. Control mice were not irradiated (●). FSA cells were injected 24 h after the final UV exposure. * and † indicate a significant difference ($p < 0.05$ and $p < 0.005$ respectively) between the control group and the groups irradiated for 3 (chart a) or 2 (chart b) weeks prior to FSA cell injection. Error bars show the SEM.

4.2.3 - Effect of UVA-I and TL01 exposure on outgrowth of FSA cells

The majority of studies on UV-enhanced tumour outgrowth have used lamps emitting wavelengths that are mostly within the UVB range. In the previous chapter, exposure of mice for 3 weeks to either the UVA or the TL01 lamps caused suppression of the CH response and loss of LC from the epidermis (section 3.2.3 and 3.2.1). To determine whether the effects of exposure to these lamps on immune parameters extended to suppression of the ability to control FSA cell outgrowth, mice were exposed to either UVA-I (340-400 nm) or TL01 (311-313 nm) twice a week for three weeks prior to FSA cell injection (section 2.1.4 and section 2.4.1).

Irradiation with 10,000 J/m² UVA-I did not increase the size of the tumours in irradiated mice compared with unirradiated controls (Figure 4.5); increasing the UVA-I dose to 500,000 J/m² (1 MED) twice a week for 3 weeks had no effect.

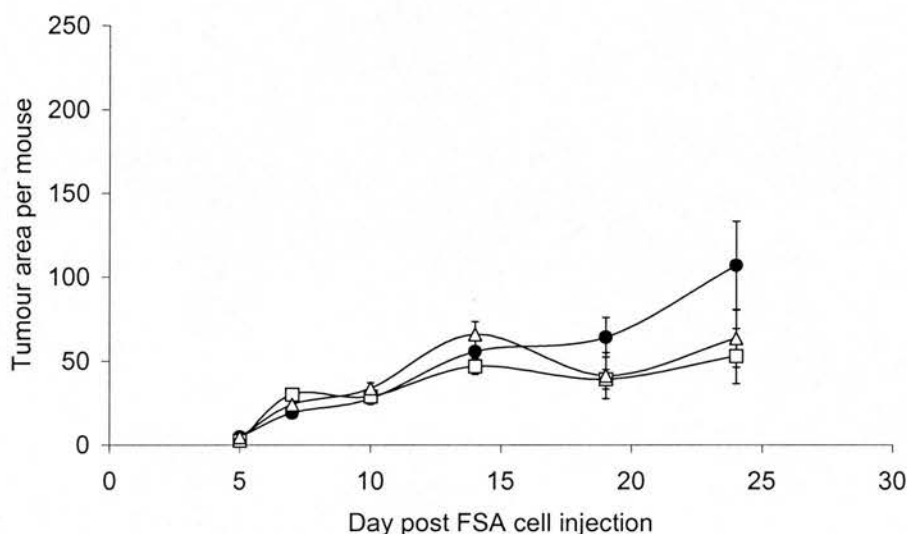


Figure 4.5. Growth of FSA cells in C3H mice following UVA-I exposure. Groups of 9 mice were irradiated twice a week for 3 weeks with 10,000 (□) or 500,000 (△) J/m² UVA-I. Control mice were not irradiated (●). FSA cells were injected 24 h after the final UV exposure. Error bars show the SEM.

Irradiation of mice for 3 weeks with 14,000 J/m² TL01 prior had no effect on the outgrowth of subsequently injected tumour cells (Figure 4.6). Continuing the irradiation protocol however, resulted in a significant increase in the size of the resulting tumours, beginning on day 18 post-injection.

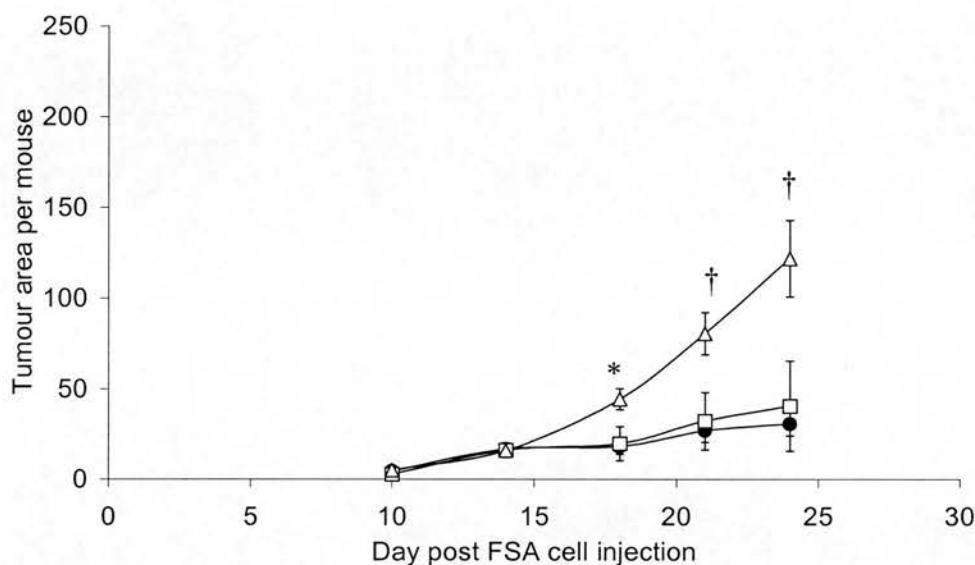


Figure 4.6. Growth of FSA cells in C3H mice following narrow-band UVB (TL01) exposure. Groups of 12 mice were irradiated twice a week for 3 weeks with 14,000 (□) J/m² TL01. Control mice were not irradiated (●). FSA cells were injected 24 h after the final UV exposure. One group continued to receive 14,000 J/m² TL01 twice a week following FSA cell injection (△). * and † show a significant difference ($p < 0.05$ and $p < 0.005$ respectively) between the unirradiated group and the group irradiated with TL01 before and after FSA cell injection. Error bars show the SEM.

4.2.4 - IL-12 and UV-enhanced FSA cell outgrowth

The effect of exogenous IL-12 (rIL-12) on UVB-enhanced tumour outgrowth was determined by irradiating mice twice a week for 3 weeks with 1500 J/m² broad-band UVB (section 2.1.4); rIL-12 was injected intraperitoneally 4 h after each irradiation

(section 2.1.6). Control groups received UVB only or no treatment. FSA cells (7.5×10^4 per site) were injected 24 h after the final irradiation (section 2.4.1).

As seen previously in section 4.2.2, mice irradiated with 1500 J/m^2 UVB showed increased outgrowth of the injected FSA cells. In mice that received rIL-12 after each injection, the size of the resulting tumours was significantly ($p < 0.05$) increased compared to unirradiated controls at all time points measured (Figure 4.7). The tumour area on irradiated rIL-12 treated mice was also greater than that on irradiated mice that had not received rIL-12 treatment; the difference between these two groups was significant ($p < 0.05$) on days 13 and 17 post-injection.

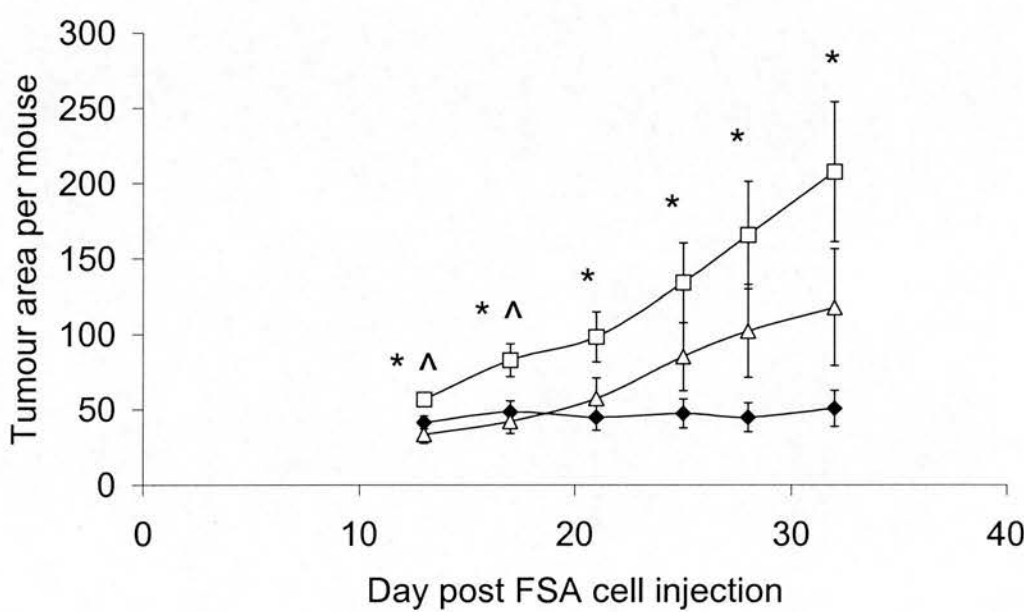


Figure 4.7. Effect of exogenous IL-12 on UVB-induced enhancement of FSA cell outgrowth. Groups of mice (11-12) were irradiated with 1500 J/m^2 UVB twice a week for 3 weeks, and received rIL-12 4 h after each irradiation (□). Control groups received broad-band UVB only (△), or were not irradiated or treated with rIL-12 prior to FSA cell injection (●). FSA cells were injected in 4 dorsal sites 24 h after the final UV exposure. * indicates a significant difference between the unirradiated control mice and the irradiated mice treated with rIL-12. ^ indicates a significant difference between the irradiated mice and the mice that were treated with rIL-12 after each irradiation. Error bars show the SEM.

4.2.5 - Effect of UVB exposure on tumour outgrowth in unirradiated skin of irradiated mice

The effect of 3 weeks exposure to broad-band UVB on the outgrowth of tumour cells in an unirradiated skin site was assessed by irradiating mice twice a week for three weeks on shaved dorsal skin with 1500 J/m^2 UVB, and injecting 7.5×10^4 FSA cells in 4 sites in the ventral skin 24 h after the final exposure, as described in section 2.4.2. Control groups received FSA cells in unirradiated dorsal skin. Whereas injection of FSA cells into irradiated skin results in increased outgrowth of the resulting tumours, irradiation of mice had no effect on the growth of tumour cells injected into unirradiated ventral skin (Figure 4.8).

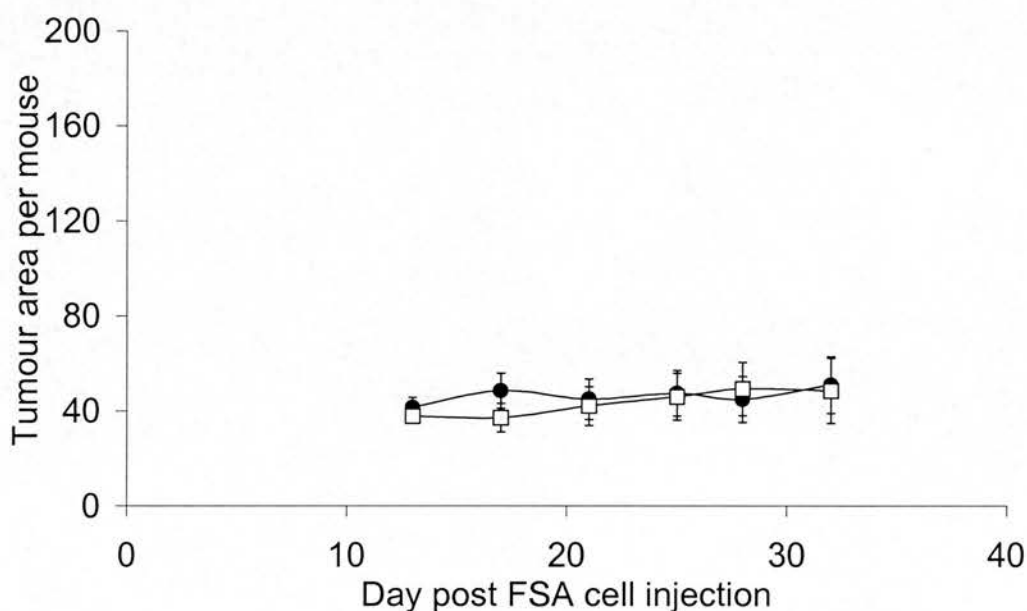


Figure 4.8. Effect of broad-band UVB exposure on outgrowth of FSA cells in a non-irradiated site. Groups of 12 mice were irradiated twice a week for 3 weeks with 1500 J/m^2 TL12. The ventral skin was shaved 24 h before the final UV exposure; FSA cells were injected in 4 ventral sites (□) 24 h after the final UV exposure. Control mice were not irradiated (●). Error bars show the SEM.

4.2.6 - Effect of UVB exposure on formation of tumours in the lung

Injection of FSA cells intravenously into the tail vein of mice, which results in the formation of colonies in the lung that can be counted by eye, has been used as a model of systemic spread of tumours (Kripke and Fidler, 1980; Milas *et al*, 1974). To assess whether sub-erythral UVB exposure affected the formation of tumours in a systemic body site that is non-skin associated, mice were irradiated twice a week for 3 weeks with 1500 J/m^2 UVB. FSA cells (7.5×10^4 per mouse) were injected into the tail vein 24 h after the final UV exposure; two weeks later, mice were killed, their lungs removed and the number of lung colonies counted after fixation (Figure 4.9, section 2.4.3).



Figure 4.9. Formation of lung tumours following intravenous injection of FSA cells. FSA cells (7.5×10^5) were injected into the tail vein of an unirradiated mouse. The lungs were removed 14 days later and stained with Bouin's fluid, such that the pale yellow tumours are easily differentiated from the surrounding tissue.

Table 4.1 shows the results of 4 separate experiments. The number of colonies produced in the lungs varied greatly between mice within a single group. No consistent effect of UV exposure was seen on the number of colonies formed in the lungs of irradiated mice. In experiments 1 and 4, the number of lung colonies was decreased in irradiated mice. However, in experiment 2, the number of lung colonies was increased in UV-exposed mice; in experiment 3, the number of colonies formed in the lungs of irradiated mice was unchanged from that in unirradiated controls.

Experiment	Mean number of lung colonies per mouse (range)	
	Control	UV
1	11 (1-31)	4 (0-9)
2	36 (7-108)	94 (7-200)
3	18 (0-105)	18 (3-72)
4	41 (6-168)	6 (1-20)

Table 4.1. Effect of broad-band UVB exposure on formation of lung colonies by FSA cells. Groups of mice (6-14) were irradiated twice a week for 3 weeks with 1500 J/m² broad-band UVB. FSA cells were injected intravenously 24 h after the final UV exposure. The lungs were removed 14 days later and stained using Bouin's fluid. Numbers in brackets show the range.

4.3 - Discussion

4.3.1 - Growth of FSA cells following injection into the skin of C3H mice

The tumour cells used in this study were from a fibrosarcoma induced by methylcholanthrene treatment of C3H mice; FSA cells from this tumour are moderately immunogenic. Immunisation of C3H mice with these cells results in development of tumour-specific T helper activity (Howie and McBride, 1982); the intratumoral host cell

populations have been well characterised (McBride *et al*, 1992) and consist mainly of macrophages, with a small component of CD4⁺ and CD8⁺ cells. The induction of an immune response to the FSA cells is dependent on the number of cells injected, such that if too few or too many are injected, tolerance occurs (McBride and Howie, 1986). In the present study, mice received varying doses of FSA cells to ascertain the number of cells that would produce tumours in at least 50% of the inoculation sites. No tumours were seen following injection of 10³ cells per site, and injection of 10⁵ cells resulted in tumours in all inoculated sites. A dose of 7.5x10⁴ cells per site was chosen for use in all further experiments, such that tumours would be induced without the use of a high dose of cells that might overwhelm any enhancing effect of UV exposure on tumour growth.

4.3.2 - Effect of broad-band UVB exposure on outgrowth of FSA cells

Previous studies have shown that antigenic UV-induced murine tumours, including fibrosarcomas and melanomas, grow progressively upon transplantation into UV-irradiated hosts. In the current study, irradiation of mice twice a week for three weeks with 1200 J/m² broad-band UVB (280-360 nm) resulted in enhanced outgrowth of FSA cells injected into the UV-irradiated skin. There may be a minimal dose requirement as 1000 J/m² had no effect. A minimal dose requirement for UV-enhanced outgrowth of K1735 mouse melanoma cells was demonstrated by Romerdahl *et al* (1988). Exposure of mice twice a week for four weeks to 5640 J/m² (FS40 sunlamps) caused increased outgrowth of melanoma cells injected intrapinnally; however, doses of 2820 J/m² and below had no effect. The decreased dose requirement in the present study compared to that study (1200 J/m² versus 5640 J/m²) could be due to a number of factors such as the differences in UV source, tumour site, cell type and cell numbers used (10⁵

per site in that study, as opposed to 7.5×10^4 here). Additionally, Romerdahl *et al* injected the melanoma cells into the irradiated ears of unshaved mice; the larger surface area of the shaved mice exposed to UVB in the present study could have reduced the actual dose required for increased tumour cell outgrowth.

Donawho *et al* (1994) looked at the relationship between UV-induced erythema and UV-enhanced tumour melanoma outgrowth, and found that doses sufficient to cause erythema in C3H mice did not cause increase outgrowth of implanted tumours in these mice. Increased tumour outgrowth in that study only occurred following multiple exposures of mice to UVB doses that were many times 1 MED. In the present study however, enhanced outgrowth of FSA cells was seen following irradiation of C3H mice with doses of UVB that do not cause erythema in these mice.

In the study by Romerdahl *et al* (1988), irradiation of mice twice a week beginning immediately after melanoma cell injection caused increased outgrowth in previously unirradiated mice. In the present study, continued exposure of mice to 1200 J/m^2 UVB after tumour injection did not further increase tumour outgrowth compared with mice that were irradiated prior to, but not following, FSA cell injection. Thus, in the FSA model, increased outgrowth of FSA cells in irradiated mice appears to depend mostly on effects of UV on mice prior to FSA injection.

Exposure of mice to a dose of 1500 J/m^2 twice over a single week prior to FSA cell injection had no effect on tumour outgrowth compared with unirradiated controls. In one experiment, exposure of mice to UVB for two weeks prior to FSA cell injection caused increased tumour outgrowth, although to a lesser extent than seen following 3 weeks UVB exposure. However, when this experiment was repeated, exposure of mice to UVB for two weeks prior to FSA cell injection significantly decreased tumour

growth compared with controls. Slight differences in the timing of the irradiation, the length of time between the final irradiation and FSA cell injection and age of the mice may have given these variable results. Exposure of nude mice to lower doses of UVB than those required for increased outgrowth of melanomas, for 4 weeks prior to melanoma cell injection resulted in decreased incidence of melanomas compared to control mice (Donawho and Kripke, 1991). It is possible that certain doses of UV may suppress the *in vivo* growth of tumour cells in a non-immunological, non-specific way, or through activation of some immune parameters such as mast cell degranulation, and release of histamine and prostaglandins.

4.3.3 - Effect of UVA-I and TL01 exposure on outgrowth of FSA cells

In previous reports investigating the effects of UVA radiation on immune responses, a variety of sources and filters have been used, making it difficult to compare one study with another. In the present study, wavelengths below 340 nm were excluded by a Cation X screen. It was shown that prior exposure to the UVA-I lamp had no effect on the growth of the FSA tumours. This supports the findings by Aubin *et al* (1991) that prior exposure of mice to UVA does not result in progressive growth of subsequently implanted melanomas. In a previous study, exposure of BALB/c mice to FS40 Sunlamps increased the growth of subsequently implanted UV-induced fibrosarcomas (De Fabo and Kripke, 1980). Use of filters that removed 99% of wavelengths below 275 nm, or 99% of wavelengths below 315 nm, demonstrated that the most effective wavelengths for UV-enhanced tumour outgrowth most probably lay within the UVB range. A study by de Gruijl *et al* (1993) demonstrated that for induction of skin cancer in hairless mice, the most effective wavelength was 293 nm, which lies within the UVB range; however,

wavelengths within the UVA range were also effective, although to a much lesser extent. In a model using the opossum *Monodelphis domestica*, exposure to UVA (over 320 nm) 3 times a week for 87 weeks gave rise to non-melanoma skin tumours in the UV-irradiated sites in 50% of the animals (Ley, 2001).

It is possible that the dose of UVA used was not high enough to cause suppression of anti-tumour responses; alternatively it may be that, although UVA can induce tumours, these wavelengths do not initiate the suppressive events required for increased tumour cell outgrowth. The UV-induced tumours that are reported as highly antigenic were all induced by exposure to wavelengths in the UVB range (Kripke, 1974); it is not known whether UVA-induced tumours demonstrate the same degree of antigenicity.

The TL01 lamp has an output mainly within the UVB range, with a peak (51%) at 311 nm. Exposure of mice to 14,000 J/m² TL01 for 3 weeks prior to inoculation with FSA cells had no effect on the outgrowth of the subsequent tumours; however, in mice that continued to be irradiated after tumour cell injection, the resulting tumours were significantly increased in size compared with those in unirradiated mice. It is not clear whether this effect of the TL01 exposure was on the FSA cells themselves, or on the host. Exposure of mice to a wider range of doses of TL01 prior to FSA cell injection would have clarified whether the increased tumour outgrowth was due to an effect on the mice.

Despite the ability of both the UVA and TL01 lamps to decrease LC numbers to a similar extent as the broad-band UVB lamp as reported in Chapter 3, this does not correlate with an effect on outgrowth of FSA cells in irradiated mice in the current study. In a study by Grabbe *et al* (1991), epidermal LC that had been pulsed with tumour

antigen (TA) prior to injection into mice, protected mice from growth of subsequently implanted tumours. However, Cavanagh *et al* (1997) demonstrated that although TA-pulsed LC could induce a DTH response to subsequently injected tumour cells, they did not induce protection against the outgrowth of injected tumour cells.

Halliday *et al* (1991) showed that UV exposure of mice before and after injection of tumour cells significantly decreased the number of LC in the epidermis above and adjacent to the tumour. However, whereas a non-immunogenic tumour attracted numerous LC in and around the tumour, significantly less LC were present in and around an immunogenic tumour, questioning the importance of LC in anti-tumour immunity. In a study using FS40 sunlamps, exposure of mice to doses that were sufficient to reduce epidermal LC numbers did not cause enhanced melanoma outgrowth (Donawho and Kripke, 1992).

It is possible that, as the tumours are injected subcutaneously, and therefore below the epidermis, LC are not the most important APC for induction of immunity to the tumours. Immunisation of mice with TA-pulsed dermal cells gave protection against the growth of subcutaneously injected tumour cells; this effect was dependent upon Ia⁺ dermal cells (Campton *et al*, 2000). Other candidate APCs for induction of tumour immunity are LN dendritic cells. As demonstrated in Chapter 3, the number of DC is decreased in LN draining the irradiated skin of mice exposed to broad-band UVB for 3 weeks. It would be interesting to assess DC numbers in LN draining the irradiated skin of mice exposed to UVA-I, or TL01 for 3 weeks to demonstrate whether they correlated with FSA cell outgrowth in irradiated mice.

Exposure to either the UVA or TL01 lamps for 3 weeks suppressed the CH response to oxazalone in C3H mice, as noted in Chapter 3; however, this did not

correlate with the ability to cause increased FSA cell outgrowth. The opposite effect was seen by Donawho and Kripke (1992), such that a 4 week irradiation of C3H mice with FS40 sunlamps, which increased the outgrowth of implanted melanomas, did not result in suppression of the CH response. Thus the CH assay, which is commonly used to assess UV-induced immunosuppression, is perhaps not a good model for the effects of UV on immune responses in skin cancer. Another useful model for UV-induced immunosuppression is the DTH assay. However, it has been demonstrated that, although treatment of C3H mice with plant poly/oligosaccharides overcomes UVB-induced suppression of DTH to alloantigen, this treatment does not prevent UVB-induced suppression of the ability to reject highly antigenic tumour cells (Strickland *et al*, 2001). Using models of cell-mediated immunity such as DTH and CH responses to investigate the mechanisms of UV-induced immunosuppression may not yield answers that are relevant to suppression of tumour immunity. As will be described in the next chapter, no DTH response to FSA cells could be detected in mice bearing FSA tumours; therefore this could not be used as a model for detecting UV-induced suppression of anti-tumour responses.

4.3.4 - IL-12 and UV-enhanced FSA cell outgrowth

In the present study, treatment with rIL-12 concomitantly with UVB exposure failed to reverse the UVB-induced increase in FSA cell outgrowth. This was unexpected for a number of reasons. First, as discussed in the previous chapter, injection of rIL-12 after UV treatment has been reported to reverse both UV-induced suppression of hypersensitivity responses, and UV-induced tolerance (Schwarz *et al*, 1996; Schmitt *et al*, 1995). Second, rIL-12 treatment has been shown to have anti-tumour effects in

murine sarcoma, adenocarcinoma and melanoma models (Nastala *et al*, 1994; Brunda *et al*, 1993). FSA cells that have been infected with a murine IL-12 expressing viral vector prior to injection into mice, show significantly decreased tumour outgrowth compared with uninfected FSA cells (Meko *et al*, 1996). Finally, *cis*-UCA induced suppression of induction of anti-tumour immunity by TA-pulsed epidermal cells can be overcome by treatment of the epidermal cells with exogenous IL-12 (Beissert *et al*, 2001).

The increase in tumour growth in mice treated with both rIL-12 and UVB seen in the present study could be due to the fact that, unlike in previous studies, the rIL-12 treatment was given prior to FSA cell injection. The inclusion of a control, unirradiated rIL-12 treated group would have given information on whether prior treatment with rIL-12 alone affects tumour cell outgrowth. Despite not including this control group, it is interesting that mice treated with rIL-12 after each UVB exposure did not show reversal of UVB-induced suppression of CH or increased FSA cell outgrowth. In the literature, IL-12 is recognised as a stimulator of immune responses, which can overcome tolerance in many different models. IL-12 failed to have an effect in one study using a mouse lung carcinoma model, where the tumours increase the numbers of granulocyte-macrophage progenitors; these granulocyte-macrophage progenitors cause immunosuppression in tumour-bearing mice. In that study, rIL-12 treatment had little effect on the immune suppression, and even increased the number of the granulocyte-macrophage progenitors (Prechel *et al*, 1995). It could be speculated that in the present study, rIL-12 treatment might increase the numbers of monocyte/ macrophages that infiltrate the UV-irradiated skin to induce immune suppression and tolerance induction (Hammerberg *et al*, 1996).

4.3.5 - Growth of FSA cells in unirradiated skin of UVB-irradiated mice

When two mice, one irradiated and one unirradiated, were joined parabiotically, subsequently implanted tumours grew progressively in both mice (Fisher and Kripke, 1977), suggesting that the effect of UV exposure on tumour outgrowth is systemic, capable of acting at sites distant from the irradiated skin. In the present study, mice were irradiated over a period of 3 weeks with 1500 J/m^2 UVB, a dose sufficient to enhance the outgrowth of FSA cells in the irradiated site. Following injection of FSA cells into the unirradiated ventral skin of irradiated mice, there was no increase in the outgrowth of the subsequent tumours compared to those in unirradiated control mice. The dose of UVB received by the mice may not have been high enough to have systemic effects; in the study by Fisher and Kripke (1977) mice were irradiated for three months ($10,000 \text{ J/m}^2$, three times a week) prior to being joined to unirradiated mice.

Alternatively, the lack of a systemic effect of UV exposure in the present study could be related to the length of time between the final irradiation and injection of the FSA cells. Mice sensitised with a contact sensitizer on irradiated skin immediately after a four day sub-erythral UV exposure have suppressed a CH response; however, irradiated mice sensitised on unirradiated skin do not exhibit UV-induced suppression of CH unless they are sensitised 3 days after the final irradiation (Shimizu and Streilein, 1994a). Mice irradiated for 12 h with a single UV dose showed increased outgrowth of an implanted FSA tumour in irradiated skin, but only if the tumour was implanted at least 48 h after irradiation. The results in section 4.2.2 demonstrated that for consistent UVB-enhanced outgrowth of FSA cells in mice irradiated with 1500 J/m^2 twice a week, at least 3 weeks exposure is required. Two weeks exposure did not have a consistent effect on outgrowth of FSA cells in the irradiated site, which could make the final

exposure in this low-dose protocol, and therefore the timing of injection of FSA cells at a distant, unirradiated site after this final exposure, quite critical for UV-enhanced tumour outgrowth. On the other hand, it may be simply that the effect of UV exposure on FSA cell outgrowth in this study is a local one; UV-enhanced outgrowth of melanomas in irradiated mice only occurs in the UV-exposed site, and is thought to be due to suppression of effector cells in the irradiated skin (Romerdaal *et al*, 1988; Donawho *et al*, 1996).

4.3.6 - Effect of UVB on formation of tumours in the lung

Injection of FSA cells intravenously into the tail vein results in the development of tumours in the lung. This has been used as a model of metastasis formation of fibrosarcomas; exposure of mice to high doses (7800 J/m²) for 6 weeks prior to FSA cell injection increases the number of lung colonies compared with unirradiated mice (Kripke and Fidler, 1980). In the present study, the UVB protocol used did not have a consistent effect on the number of lung colonies formed, such that numbers were decreased, increased, or unchanged from those of unirradiated controls. Tail vein injections are fairly time consuming, and during this time clumps tended to form within the FSA cell suspension, probably due to the release of DNA from dead cells. To overcome this, DNase was added to the cell suspension (section 2.1.3). Additionally, only a small volume of cells (100µl) was injected into the tail vein; small differences in the actual volume received by the mice could have had affected the number of lung tumours formed.

4.4 - Summary of Chapter 4

Exposure of mice to sub-erythral doses (1200 J/m^2) of broad-band UVB induced enhanced outgrowth of FSA cells injected subcutaneously into the irradiated skin. This effect was not seen following 1 or 2 weeks of exposure, but required an irradiation protocol lasting at least 3 weeks. Continuing the broad-band UVB exposure after FSA cell injection did not further increase the UV-enhanced tumour outgrowth seen in mice irradiated prior to FSA cell injection. Therefore exposure to UVB reduces the ability of the mice to control FSA cell outgrowth.

Despite the immunosuppressive effects of the UVA and TL01 lamps, exposure of mice for 3 weeks to either of these wavebands had no effect on the outgrowth of subsequently injected FSA cells. Thus UV-enhanced FSA cell outgrowth is not correlated with loss of epidermal LC or suppression of CH. Continued exposure to TL01 after FSA cell injection resulted in a significant increase in the size of the outgrowing tumours; perhaps the UV wavelengths emitted by this lamp act directly on the *in vivo* FSA cells to stimulate growth, rather than affecting the ability of the mouse to control tumour growth.

Injection of FSA cells into unirradiated skin of UVB-irradiated mice did not result in the enhanced tumour outgrowth seen following injection into irradiated skin. It could be that the dose of UVB used was not high enough; alternatively, UVB may only act locally on irradiated skin to enhanced tumour growth.

Exogenous IL-12, which can stimulate anti-tumour immune responses, and overcome UV-induced tolerance, was injected into mice after each irradiation of a 3 week UVB protocol. IL-12 treatment in this study did not reverse the UVB-enhanced FSA cell outgrowth. Interestingly, IL-12 treatment further increased the enhanced FSA

growth seen in irradiated mice; it may be that IL-12 potentiates the UV-induced suppression of anti-tumour immune responses in this system.

Chapter 5

Effect of broad-band UVB exposure prior to FSA cell injection on immune responses in tumour-bearing mice

5.1 - Introduction

As described in Chapter 4, exposure of mice to broad-band UVB over a 3 week period caused increased outgrowth of subsequently injected FSA cells. Using 3 different UV sources, it was demonstrated that enhanced tumour outgrowth was not related to UV-induced reduction of epidermal LC numbers or suppression of CH. It is possible that UV exposure caused the release of substances in UV-irradiated skin that directly stimulate the growth of the injected FSA cells; however, it is more likely that the UV radiation increases tumour outgrowth by suppression of immune responses to the tumour cells. Previous studies have shown that transfer of immune cells from irradiated tumour-bearing mice to naïve mice results in increased outgrowth of implanted tumours in the recipient mice (Fisher and Kripke, 1977), and that UV exposure only causes increased outgrowth of antigenic tumours; outgrowth of non-antigenic tumours is not affected by prior UV irradiation (Kripke and Fisher, 1976). In the current chapter, the effect of prior UVB exposure on immune responses in tumour-bearing mice is investigated.

The FSA cells used in the present study are moderately immunogenic, therefore initially an attempt was made to develop a way to measure anti-tumour responses in mice that have been injected with live FSA cells. DTH responses of tumour-bearing mice to FSA cells were compared with those of naïve mice. Additionally, the *in vitro*

proliferation of LN cells from tumour-bearing mice in response to FSA cell antigens was tested.

In Chapter 4, the effect of UVB on tumour outgrowth was demonstrated to be a local one, as injection of FSA cells into a site distant from the UVB-exposed skin of irradiation of mice had no effect on tumour outgrowth. Therefore, in the present chapter, immune parameters of tumour-draining LN from irradiated mice bearing FSA tumours were compared with those of LN from unirradiated tumour-bearing mice, and control, tumour-free mice.

The number of cells in the local LN draining the site of tumour cell injection was counted in control and irradiated mice, at various time points following inoculation of the FSA cells. The spontaneous proliferation of these DLN cells was also measured.

In a study of breast cancer patients, it was demonstrated that the phytohaemagglutinin (PHA)- induced proliferation of peripheral blood mononuclear cells from cancer patients was decreased compared with that of peripheral blood mononuclear cells from healthy controls (Wiltschke *et al*, 1995). In the present study, the proliferation of cells from LN draining the site of tumour injection in response to Con A and PHA was measured; responses of cells from irradiated and unirradiated tumour-bearing and tumour free mice were compared.

The expression of MHC Class II, Thy 1.2, CD4 and CD8 by DLN cells from irradiated and unirradiated tumour-bearing mice was also measured by flow cytometry at various points during outgrowth of the FSA cells, to assess whether this was affected by FSA cell injection.

The cytokine IL-10 has been shown to promote lung cancer growth by suppressing T cell functions (Sharma *et al*, 1999) and there is also evidence that it is a mediator of

UV-induced suppression (Kurimoto *et al*, 2000). The *in vitro* production of this cytokine by DLN cells from tumour-bearing mice was measured to assess whether this cytokine has a role in the increased outgrowth of tumour cells in irradiated mice.

Tumours arising from the FSA cells in this study have previously been shown to have infiltrating host cells consisting mainly of macrophages, with a few CD4⁺ and CD8⁺ cells (McBride *et al*, 1992). An attempt was made in the present study to assess immunohistochemically whether prior UVB-exposure affected the infiltration of host immune cells into the tumours that arose from injected FSA cells.

5.2 - Results

5.2.1 - Delayed-type hypersensitivity response to FSA cells by tumour-bearing mice

To determine whether tumour-bearing mice were able to mount a DTH response to FSA cells, mice received 7.5×10^4 FSA cells either in 4 dorsal sites or intravenously. Control mice were injected with HBSS only. Fourteen days later, mice were challenged intrapinnally with irradiated FSA cells (10^5 per ear), and the ear swelling measured 24 h later as described in section 2.2.2. No difference in ear swelling was seen in mice that had had FSA cell injected dorsally compared with control mice that had not received FSA cells prior to challenge (Figure 5.1). Mice that had received FSA cells intravenously 14 days prior to challenge with FSA cells had slightly increased ear swelling compared with mice that had not received FSA cells, however this increase was not significant. The DTH response was therefore not a reliable measure of anti-tumour immune responses in tumour-bearing mice.

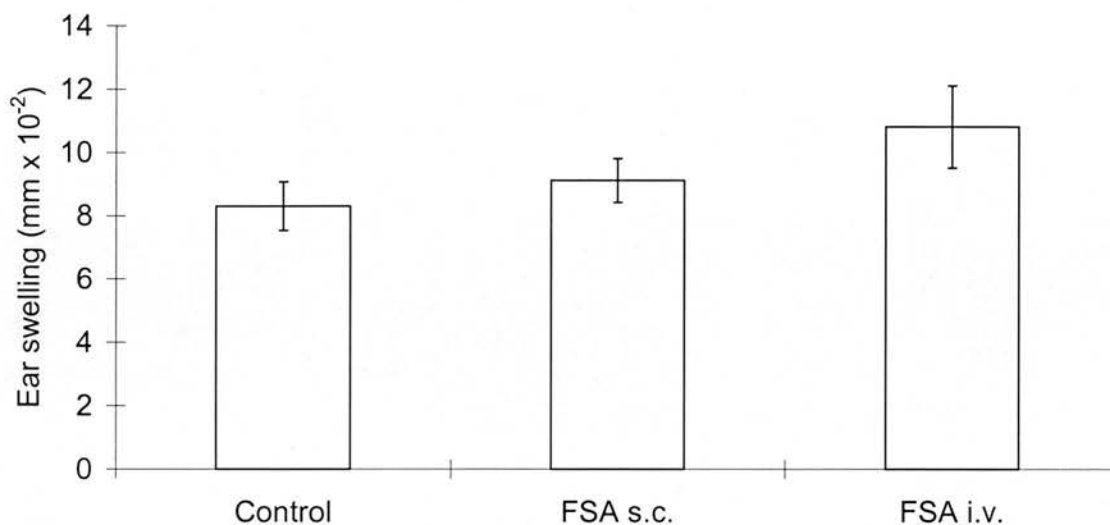


Figure 5.1. Delayed type hypersensitivity response to FSA cells. Groups of 8 mice were injected with 7.5×10^4 FSA cells in four dorsal sites (FSA s.c.) or intravenously in the tail vein (FSA i.v.). Control mice were injected subcutaneously in four dorsal sites with HBSS (Control). All mice were challenged intrapinnally with irradiated FSA cells 14 days later (10^5 cells per ear). Error bars show the SEM.

5.2.2 - *In vitro* proliferation of LN cells in response to FSA cell antigens

The *in vitro* proliferation of immune cells from tumour bearing mice in response to FSA cell antigens was compared with that of immune cells from tumour free controls. A glycine extract of FSA cell tumour antigen (TA) was prepared as described in section 2.5.4. To determine whether there was an optimum TA concentration for immune cell proliferation, live FSA cells were injected into 4 sites in the shaved dorsal skin, and mice killed at day 22 post-FSA cell injection. The LN draining the tumour site and the spleen were removed and disaggregated to give either LN or spleen cell suspensions (section 2.5.1). The cells were then cultured for 96 h alone, or in the presence of varying concentrations ($0.2 \mu\text{g protein/ml}$ to $2000 \mu\text{g protein/ml}$) of FSA cell TA. As shown in

Figure 5.2a, unstimulated proliferation of LN cells from tumour-bearing mice was higher than that of LN cells from naïve, control mice. Proliferation of cells from both groups was completely abrogated upon addition of 2000 µg/ml of FSA TA; however this was due to an effect of the glycine extraction medium, which was toxic at the highest concentration added, but otherwise had no effect on proliferation (section 2.5.4). Cells from tumour bearing mice that were cultured in the presence of 20 µg/ml of TA showed increased proliferation compared with unstimulated cells from the same mice, with a SI of 2.2 (Figure 5.2a). The proliferation of LN cells from control mice was not increased by addition of TA at any of the concentrations tested, and the cpm of LN cells from tumour-bearing mice was higher compared with that of controls following incubation in 0.2-20 µg/ml FSA TA.

Proliferation of spleen cells from the same mice is shown in Figure 5.2b. As seen with the LN cells, unstimulated proliferation of spleen cells from tumour-bearing mice was higher than that of spleen cells from control mice (not significant). Whereas the increase in proliferation of LN cells in response to FSA TA stimulation was seen only in cells from tumour-bearing mice, spleen cells from both tumour-bearing and tumour-free mice showed increased proliferation in response to stimulation with 200 µg/ml and 0.2 µg/ml TA respectively (Figure 5.2b). Due to the low number of samples used, no statistical tests could be done on these data.

The proliferation of LN cells in response to 20 µg/ml was assayed 2 more times at varying incubation times (Table 5.1). As seen in Figure 5.2a, LN cells from tumour-bearing mice showed increased unstimulated proliferation compared with cells from the LN of tumour-free mice at all time points tested in experiment 1 (Table 5.1).

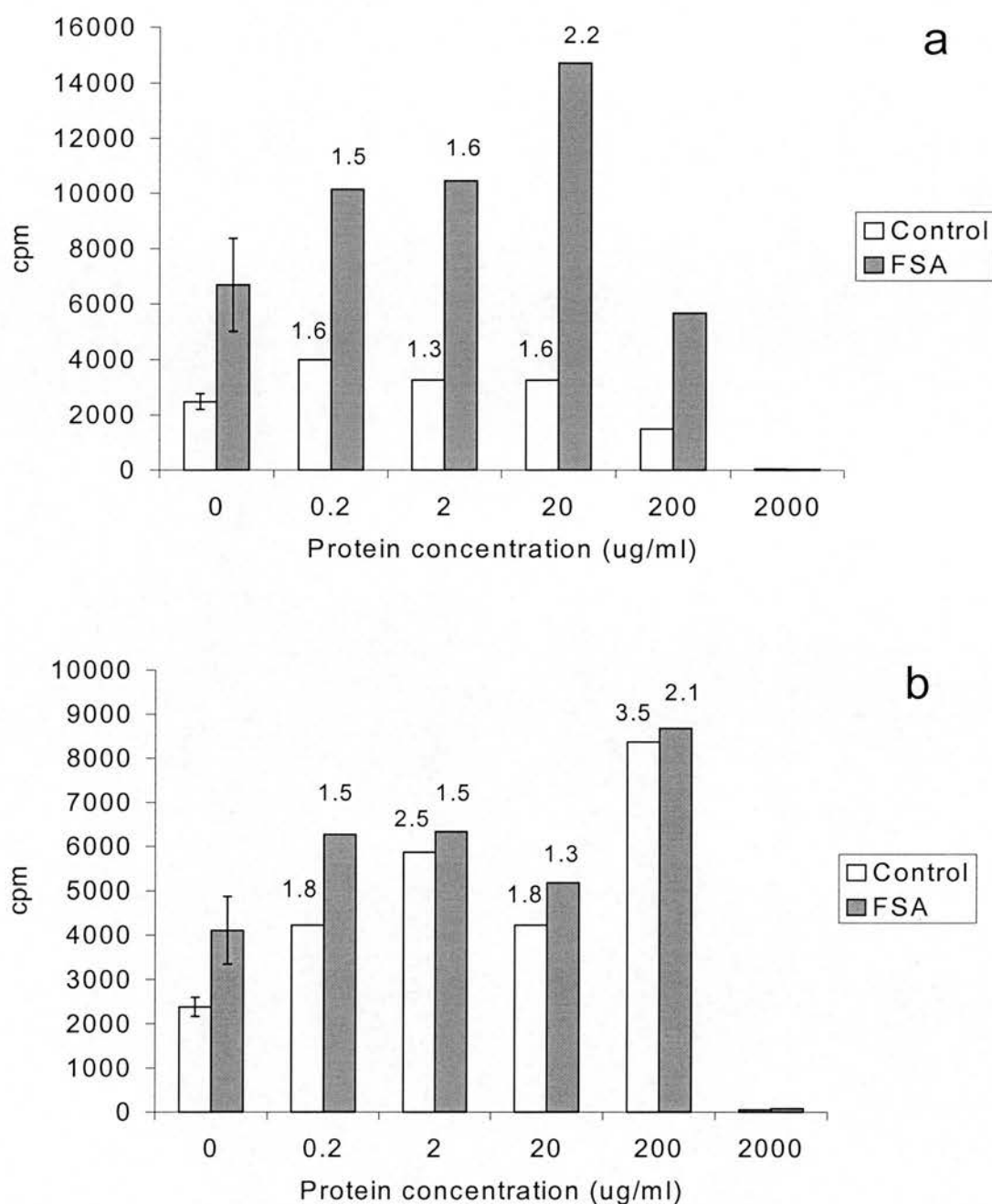


Figure 5.2. Proliferation of immune cells *in vitro* in response to varying concentrations of FSA extract. Cells from (a) LN draining the tumour site or (b) the spleen were cultured for 96 h in medium alone (0), or with varying concentrations (0.2-2000 µg/ml) of a glycine extract of FSA cells; ^3H -Thymidine was added for the final 8 h of culture. The cells were pooled from two tumour-bearing mice that had received 7.5×10^4 FSA cells in 4 dorsal sites (FSA) 20 days previously, or from two untreated aged matched controls (Control). Error bars show the SEM of 3 replicate wells. Columns without SEM show the mean of 2 replicate wells. The SI is shown above columns where proliferation was increased compared with unstimulated cells.

	Experiment 1				Experiment 2			
	RPMI		FSA TA		RPMI		FSA TA	
	Control	Tumour	Control	Tumour	Control	Tumour	Control	Tumour
48 h	476 (70)	3738 (444) *	800 (57) †	7416 (422) †\$	2813 (129)	5724 (471) *	2878 (270)	7612 (438) †\$
72 h	2055 (175)	11450 (691) *	3577 (412) †	13392 (572) \$	7609 (390)	8055 (346)	6646 (586)	9865 (418) \$
96 h	2842 (554)	25643 (3963) *	6115 (973)	28246 (998) \$	ND	ND	ND	ND
120 h	ND	ND	ND	ND	16089 (1315)	11328 (359)	14785 (1906)	16030 (3124)

Table 5.1. Proliferation of DLN cells in response to FSA extract following varying culture times. Cells from LN draining the tumour site were cultured for 48, 72, 96 or 120 h in the presence of 20 µg/ml of a glycine extract of FSA cells; ³H-Thymidine was added for the final 8 h of culture. The cells were taken from tumour-bearing mice that had received 7.5 x 10⁴ FSA cells in 4 dorsal sites (Tumour) 20 days previously, or from untreated aged matched controls (Control), and cultured alone (RPMI) or with FSA extract (FSA TA). Results in cpm from 2 separate experiments are shown. Numbers in brackets show the SEM (of 3-5 replicate wells). * indicates a significant difference (p<0.05) between unstimulated proliferation of cells from control and tumour-bearing mice. † indicates a significant difference (p<0.05) between unstimulated cells and cells incubated with FSA TA. \$ indicates a significant difference (p<0.05) between FSA TA-stimulated proliferation of cells from control and tumour bearing mice.

The level of proliferation of cells incubated with FSA TA was significantly higher for LN cells from tumour-bearing mice compared with LN cells from control mice, at all time-points tested, except one. Proliferation of LN cells from tumour-bearing mice was increased when the cells were cultured in the presence of 20 µg/ml FSA TA (Table 5.1), compared with unstimulated cells from tumour-bearing mice, although not to the same extent as the increase seen at 96 h in the previous experiment (Figure 5.2a). This increase was only significant in 2 cases; additionally, LN cells from control mice also showed significantly increased proliferation following stimulation with FSA TA in two cases, compared with unstimulated cells from control mice. Thus the antigen-specific FSA TA stimulated increase in proliferation of LN cells from tumour-bearing mice was not reproducible, and was therefore considered an unreliable test of tumour-specific responses in tumour-bearing mice.

In the following experiments, the effect of UVB exposure of mice prior to injection of FSA cells on cells of the LN (number, phenotype and *in vitro* proliferation) draining the site of tumour cell injection was investigated. Mice were exposed twice a week for 3 weeks to broad-band UVB (section 2.1.4), and FSA cells (7.5×10^4 per site) injected into 4 dorsal sites (section 2.4.1). Control groups received FSA cells only, UVB only, or no treatment. The mice (4 per group) were killed on days 9, 13 and 18 post-injection of the FSA cells. These time points were chosen because following injection of the FSA cells, at least 90 % of the mice are tumour bearing by day 9, and as the tumours grow faster and often show signs of ulceration earlier in mice that have been exposed to UVB, many of them require to be killed after 18 days. LN draining the site of tumour injection were then removed and the LN for one group pooled; LN cell number,

spontaneous and mitogen-induced proliferation of the LN cells, and cell surface marker expression were then assessed.

5.2.3 - Cell numbers in LN draining the site of FSA cell injection

Following removal of the LN, the number of LN cells per ml was estimated by counting with a haemocytometer as described in Section 2.5.1; the number of cells per LN was then calculated for each group. The results of two separate experiments are shown in Figures 5.3a and 5.3b. The number of cells/LN in unirradiated tumour-free mice remained fairly constant over the course of a single experiment, from days 9 to 18.

In the first experiment, it can be seen that in tumour-free mice, 3 weeks exposure to UVB had little effect on LN cell number compared with unirradiated control mice, with only a slight increase in number on days 9 and 13 (Figure 5.3a); this group was not included the second time the experiment was done. The results of both experiments demonstrate that the number of cells/LN of unirradiated tumour-bearing mice was around double that of tumour-free controls on day 9. LN cell numbers still remained higher in tumour-bearing mice on day 13 but had decreased from day 9, and were further decreased on day 18. In both experiments, mice that had been exposed to UVB prior to FSA cell injection showed a similar pattern of LN cell number to unirradiated tumour-bearing mice.

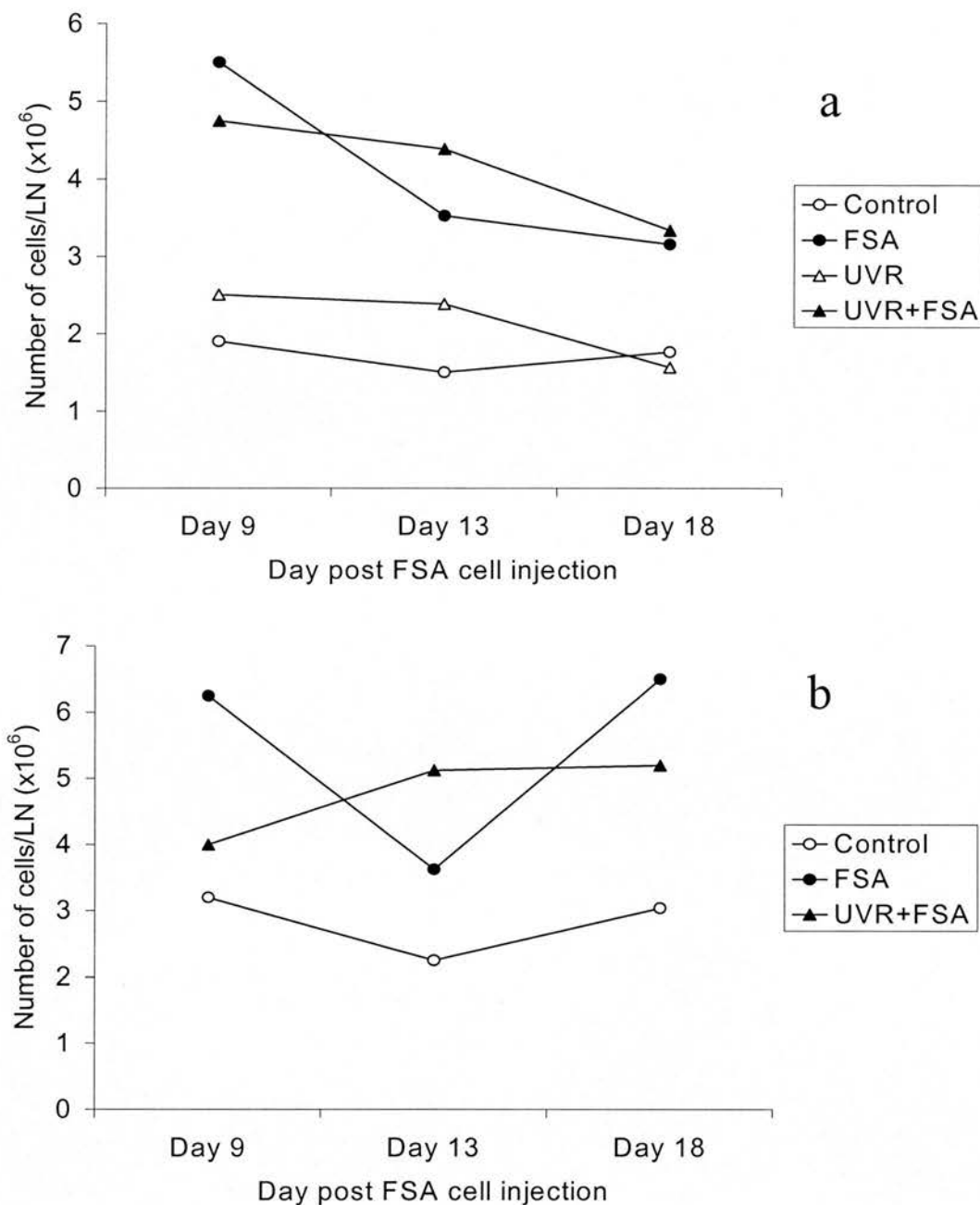


Figure 5.3. Effect of UVB exposure on DLN cell number in tumour-bearing mice.

Groups of 12 mice were irradiated twice a week for three weeks with 1500 J/m^2 UVB, and 7.5×10^4 FSA cells were injected in 4 dorsal sites 24 h after the final exposure (UVR+FSA). Control groups received FSA cells but were not irradiated (FSA), were irradiated but not injected with FSA cells (UVR), or were untreated (Control). On days 9, 13 and 18 post-FSA cell injection, 4 mice per group were killed and LN draining the tumour-bearing skin were pooled for each group. Cell number was counted by eye using a light microscope and the number of cells/LN calculated (a). This experiment was repeated (b) without the inclusion of the irradiated, tumour-free group.

5.2.4 - Spontaneous proliferation of DLN cells

As seen in section 5.2.2, the spontaneous proliferation of DLN cells taken from tumour-bearing mice on day 20 post FSA cell injection was higher than in tumour-free mice. To assess whether the increase in spontaneous proliferation of LN cells taken from mice that had been injected with FSA cells was affected by UVB exposure of the mice prior to FSA cell injection, LN cells taken from mice on days 9, 13 and 18 were cultured for 24 h as described in section 2.5.2. The results of two separate experiments are shown in Figures 5.4a and 5.4b. The spontaneous proliferation of LN cells from UVB exposed, tumour-free mice was unchanged from that of cells from unirradiated tumour-free mice on days 9 and 13 and very slightly increased on day 18 ($p < 0.05$) (Figure 5.4a). The spontaneous proliferation of LN cells from tumour-bearing mice (irradiated and unirradiated) was significantly higher than that of control tumour-free mice at all time points in both experiments (Figures 5.4a and 5.4b). However, the proliferation of LN cell from irradiated tumour-bearing mice was significantly suppressed compared with that of LN from unirradiated tumour-bearing mice at all time points, except for day 13 in experiment 2, when the level of spontaneous proliferation was similar for both groups.

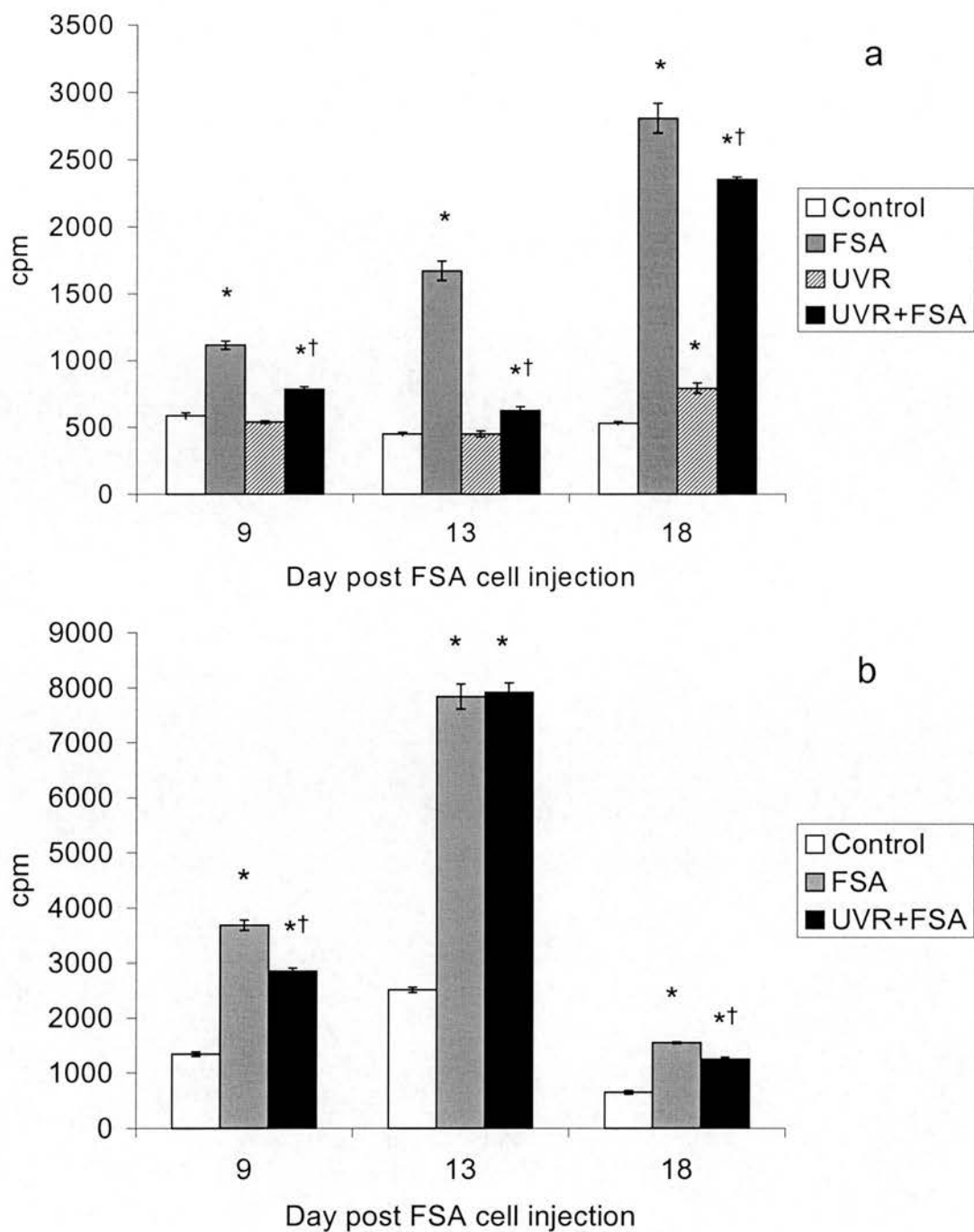


Figure 5.4. Effect of UVB exposure on spontaneous proliferation of DLN cells from tumour-bearing mice. Mice were treated as described in Figure 5.3. On days 9, 13 and 18 post-FSA cell injection, 4 mice per group were killed and LN draining the tumour-bearing skin were pooled for each group. Cells were cultured for 24 h in the presence of ^3H -Thymidine; the average for 5 replicate wells is shown (a). Error bars show the SEM. This experiment was repeated without the inclusion of the irradiated, tumour-free control group (b). * indicates a significant increase ($p < 0.05$) compared with the unirradiated tumour-free group. † indicates a significant decrease ($p < 0.05$) of irradiated tumour-bearing mice compared with unirradiated tumour-bearing mice.

5.2.5 - Mitogen-stimulated proliferation of DLN cells

In the previous section, DLN cells from unirradiated tumour-bearing mice had increased levels of spontaneous proliferation compared with tumour-free controls; this increase was not as high for DLN cells from mice that had been irradiated prior to FSA cell injection. To assess whether the ability of these cells to proliferate *in vitro* in response to mitogenic stimulation was also affected, DLN cells taken at days 9, 13 and 18 from irradiated and unirradiated tumour-bearing mice were cultured for 48 h in the presence of 2.5 µg/ml Con A or PHA as described in section 2.5.3.

The results of one experiment are shown in Table 5.2. DLN cells from tumour-bearing mice showed significantly increased background proliferation compared to cells from tumour-free mice on all days tested, with one exception (irradiated tumour-bearing mice on day 13). In general, the level of Con A-induced proliferation was also higher in tumour bearing mice than in tumour-free controls. The overall level of proliferation in response to PHA was consistently lower than that to Con A; however, DLN cells from tumour-bearing mice showed increased PHA-induced proliferation compared with those from tumour-free mice. UVB exposure did not appear to consistently affect the mitogen-induced proliferation of DLN cells from tumour-bearing or tumour-free mice.

	Day 9			Day 13			Day 18		
	RPMI	Con A	PHA	RPMI	Con A	PHA	RPMI	Con A	PHA
Control	1335 (114)	130209 (6611) 98	50722 (1127) 38	733 (56)	104750 (2730) 143	28651 (1076) 39	842 (57)	109555 (3840) 130	24296 (1843) 29
FSA	2871 (242)	130724 (2211) 46	68352 (880) 24	2523 (209)	143093 (3360) 57	63530 (1673) 25	2623 (56)	137504 (3961) 52	71555 (1878) 27
UVR	1571 (136)	123474 (2095) 79	54664 (2801) 35	449 (25)	79493 (3383) 177	23744 (878) 53	1053 (70)	104738 (2405) 99	35461 (799) 34
UVR+FSA	2645 (210)	121485 (2485) 46	78546 (744) 30	742 (74)	79230 (3042) 107	37745 (1396) 51	4221 (237)	123606 (3886) 29	57860 (1782) 14

Table 5.2 Proliferation of DLN cells from irradiated and unirradiated tumour-bearing mice in response to mitogens. Mice were treated as described in Figure 5.3. On days 9, 13 and 18 post-FSA cell injection, 4 mice per group were killed and LN draining the tumour-bearing skin were pooled for each group. Cells were cultured for 48 h alone (RPMI), or in the presence of concanavalin A (Con A) or phytohaemagglutinin (PHA); ³H-Thymidine was added for the final 8 h of culture. Numbers are expressed as the mean cpm (SEM) of 4 replicate wells. The SI is shown in blue. This experiment was repeated with similar results.

5.2.6 - IL-10 production by DLN cells

The release of IL-10 by DLN cells taken from mice on day 22 post FSA cell injection and cultured *in vitro* was assessed as described in section 2.5.5. Following culture for 24 h of unstimulated LN cells from untreated control mice, IL-10 was not present in the culture supernatant in detectable amounts (more than 4 pg/ml) (Table 5.3). After 48 h culture, IL-10 could be detected in the supernatant, and the level of IL-10 was increased when cells had been cultured for 96 h.

Cells from tumour-bearing mice produced detectable levels of IL-10 after only 24 h culture, which continued to increase after 48 h and 96 h culture (Table 5.3). The level of IL-10 in culture supernatants of DLN cells after 24 h and 48 h was similar for both UVB exposed and unirradiated mice; at 96 h, IL-10 levels were higher in the culture supernatant from irradiated tumour-bearing mice, compared with unirradiated tumour bearing mice.

Culture time (h)	Concentration of IL-10 in culture supernatant (pg/ml)		
	Control	FSA	UVR+FSA
24	< 4	97	73
48	64	271	209
96	986	1290	1771

Table 5.3. IL-10 production by cultured LN cells. LN were removed from irradiated (UVR+FSA) or unirradiated (FSA) tumour-bearing mice, or from tumour-free controls (Control), on day 22 post FSA cell injection. Cells were cultured for 24, 48 and 96 h and the supernatants tested by ELISA for the presence of IL-10 protein. Values are expressed as the mean of two replicate wells.

5.2.7 - Phenotyping of DLN cells

The percentages of DLN cells expressing Thy 1.2, CD4, CD8 and MHC class II were assessed by flow cytometry, as described in section 2.5.6. The levels for the total cell population were measured because there are very few cells in the larger, granular population, and the expression of a particular marker by the whole cell population was similar to that by either the lymphocyte population or the larger, more granular cells. The results of two separate experiments are shown in Tables 5.4-5.7; due to the variability of the technique, the results for the two experiments could not be combined. As the experiments were only done twice, the statistical significance of the trends could not be evaluated.

Thy 1.2 was found on 55-78 % of DLN cells from untreated mice (Table 5.4); levels were similar for both irradiated and unirradiated tumour-free mice. At all timepoints after FSA cell injection, the percentage of Thy 1.2⁺ cells decreased in unirradiated tumour-bearing mice, compared with untreated mice. Thy 1.2 expression by DLN cells from UVB exposed tumour-bearing mice also decreased generally compared with that in untreated mice, although not to the same extent as in unirradiated tumour-bearing mice.

CD4 was found on 32-49 % of DLN cells from untreated mice (Table 5.5); CD4 expression by DLN cells from UVB exposed tumour-free mice was slightly increased on day 9, and slightly decreased on days 13 and 18, compared with unirradiated controls. At all time points in both experiments, except one, the percentage of CD4⁺ cells was decreased in the unirradiated tumour-bearing mice, compared with untreated mice however, CD4 expression by DLN cells from irradiated tumour-bearing mice was not consistently decreased compared with untreated animals.

Day post FSA cell injection	Experiment	Control	FSA	UVR	UVR+FSA
9	1	55	46	60	50
	2	77	71	ND	72
13	1	67	52	67	63
	2	78	72	ND	76
18	1	56	50	56	59
	2	73	70	ND	73

Table 5.4. Percentage of LN cells positive for Thy 1.2. LN were removed from irradiated (UVR+FSA) or unirradiated (FSA) tumour-bearing mice, or from irradiated (UVR) or unirradiated (Control) tumour-free controls on day 9, 13 and 18 post FSA cell injection. LN cells were stained for the presence of Thy 1.2, and analysed by flow cytometry.

Day post FSA cell injection	Experiment	Control	FSA	UVR	UVR+FSA
9	1	32	30	34	34
	2	49	42	ND	45
13	1	43	35	39	39
	2	48	43	ND	45
18	1	36	22	32	32
	2	45	45	ND	45

Table 5.5. Percentage of LN cells positive for CD4. LN were removed from irradiated (UVR+FSA) or unirradiated (FSA) tumour-bearing mice, or from irradiated (UVR) or unirradiated (Control) tumour-free controls on day 9, 13 and 18 post FSA cell injection. LN cells were stained for the presence of CD4, and analysed by flow cytometry.

CD8 was found on 11-24 % of DLN cells from untreated mice (Table 5.6); CD8 expression by DLN cells from irradiated tumour-free mice was slightly increased

generally compared with unirradiated mice. Tumour-cell injection had little effect on CD8⁺ cell numbers whether the mice had been irradiated or not.

Day post FSA cell injection	Experiment	Control	FSA	UVR	UVR+FSA
9	1	18	16	21	16
	2	23	22	ND	20
13	1	24	18	26	23
	2	19	21	ND	19
18	1	11	13	11	13
	2	24	20	ND	23

Table 5.6. Percentage of LN cells positive for CD8. LN were removed from irradiated (UVR+FSA) or unirradiated (FSA) tumour-bearing mice, or from irradiated (UVR) or unirradiated (Control) tumour-free controls on day 9, 13 and 18 post FSA cell injection. LN cells were stained for the presence of CD8, and analysed by flow cytometry.

MHC class II (Ia) was found on 5-19 % of DLN cells from untreated mice (Table 5.7); MHC class II expression by DLN cells from irradiated tumour free mice was similar to that of untreated controls. At all timepoints after FSA cell injection, the number of MHC class II⁺ DLN cells from unirradiated, tumour-bearing mice was increased compared with tumour-free controls. In the first experiment, the percentage of DLN cells expressing MHC class II from UVB exposed, tumour-bearing mice was not increased until day 18 post-FSA cell injection. In the second experiment, the percentage of MHC class II⁺ DLN cells from irradiated tumour-bearing mice was slightly increased on days 9 and 13, although not as much as that of DLN cells from unirradiated tumour-bearing mice. The histograms of MHC class II expression by DLN cells from

unirradiated tumour-free and tumour-bearing mice, and irradiated tumour-bearing mice (Experiment 1, day 13 post FSA cell injection) are shown (Figure 5.5). It can be seen that the intensity of MHC class II expression was increased on DLN cells from unirradiated tumour-bearing mice, but not from irradiated tumour-bearing mice, compared with DLN cells from control, tumour-free mice.

Day post FSA cell injection	Experiment	Control	FSA	UVR	UVR+FSA
9	1	5	7	3	6
	2	19	27	ND	24
13	1	7	13	7	7
	2	18	25	ND	23
18	1	17	27	15	24
	2	ND	ND	ND	ND

Table 5.7. Percentage of LN cells positive for MHC class II. LN were removed from irradiated (UVR+FSA) or unirradiated (FSA) tumour-bearing mice, or from irradiated (UVR) or unirradiated (Control) tumour-free controls on day 9, 13 and 18 post FSA cell injection. LN cells were stained for the presence of MHC class II, and analysed by flow cytometry.

5.2.8 - Detection of tumour-infiltrating cells

Many tumours have an infiltrating population of immune cells, such as macrophages and T cells (Zhang *et al*, 1998; Hakansson *et al*, 1997); tumours arising from the FSA used in the present study have previously been shown to have mostly infiltrating macrophages, with a few CD4⁺ and CD8⁺ cells (22%, 5% and 3% respectively) (McBride *et al*, 1992). In the present study, an attempt was made to assess

the prevalence of tumour-infiltrating cells, by flow cytometric analysis of the cells from disaggregated tumours.

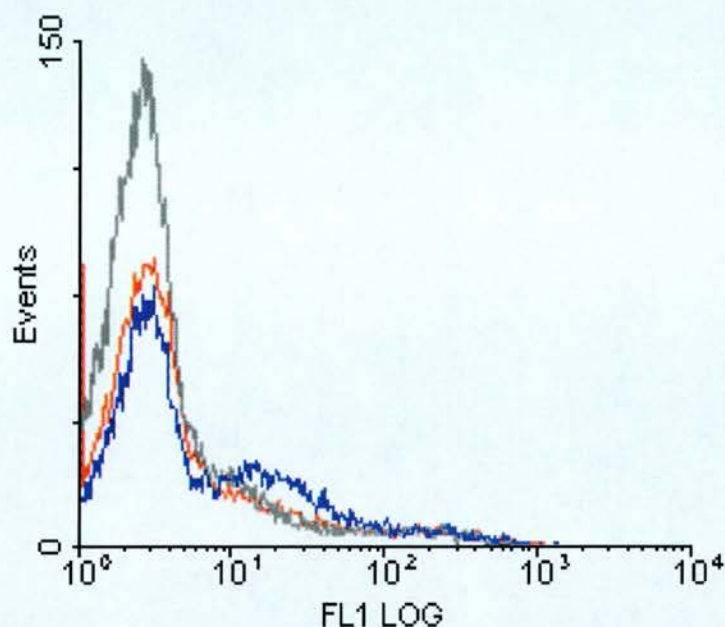


Figure 5.5. Expression of MHC class II by LN cells from control and tumour-bearing mice. LN were removed on day 9, 13 and 18 post FSA cell injection, and the LN cells were stained for the presence of MHC class II and analysed by flow cytometry. The fluorescence intensity of the total cell population is shown for unirradiated tumour-free controls (grey line), unirradiated tumour-bearing mice (blue line) and irradiated tumour-bearing mice (orange line), on day 13 post-FSA cell injection.

Mice were irradiated twice a week for 3 weeks prior to FSA cell injection. Tumours were taken from these and unirradiated tumour-bearing control mice on day 18 and 24 post FSA cell injection. Tumours were disaggregated by incubation in Dispase as described in section 2.6.1, and the expression of Thy 1.2, CD4, CD8 and CD11b

analysed by flow cytometry. Following disaggregation of tumours in Dispase, cells expressing Thy 1.2 (7-35 %), CD4 (7-24%) and CD11b (14-38%) could be detected, however, no CD8⁺ cells were observed. An alternative method of enzymatic digestion was tested to determine whether expression of the CD8 cell surface marker was affected by digestion in Dispase. Mice were irradiated twice a week for 3 weeks prior to FSA cell injection. Tumours were taken from these and unirradiated tumour-bearing control mice on day 24 post FSA cell injection. Tumours from one mouse per group were excised, cut into fragments and incubated for 1 h at 37°C in 3 ml PBS containing 0.1% (w/v) collagenase (Sigma) and 20µg/ml DNase. Single cell suspensions were prepared by mechanical disaggregation through a nylon cell strainer, washed twice, counted, and expression of cell surface markers determined by flow cytometry, as described in section 2.5.6. Cells expressing Thy 1.2 (10-13%), CD4 (20-21%) and CD11b (27-38%) could be detected, however, no CD8⁺ cells were observed using this method of disaggregation.

It could be that only low numbers of CD8⁺ cells infiltrate the FSA tumours, such that they were undetectable, alternatively it is possible that the enzymatic digestion of the tumour tissue affected levels of expression of the CD8 molecule. It was also found that disaggregation of tumours which were removed earlier than day 18 post FSA cell injection generally resulted in a low cell recovery (around 2×10^5 cells from 4 tumours), due to the smaller size of the tumours. Therefore, an immunohistochemical technique was developed in an attempt to visualise both infiltrating cells at earlier time points, and any CD8⁺ cells.

The suitability of antibodies against CD4, CD8, Thy 1.2 and CD11b and MHC class II for use in immunohistochemistry was first tested using spleen and LN sections from normal mice, as outlined in section 2.6.2. After testing at a range of dilutions, only

CD4 and CD8 antibodies were found to give positive staining; Figure 5.5 shows examples of normal LN stained with antibodies to CD4 (Figure 5.6a) and CD8 (Figure 5.6b). These antibodies were then used at a range of dilutions on sections of a FSA tumour taken from an otherwise untreated mouse, 22 days after tumour cell injection (Figure 5.7). There was some background staining on the tumour sections, which could not be eliminated (Figure 5.7b). Attempts to eliminate this staining, which were unsuccessful, included increasing the concentration/length of the peroxidase blocking step; titration of the secondary (biotinylated goat anti-rat IgG) antibody; raising the pH to 9.0 to reduce avidin binding to any charged sites; and blocking of any endogenous biotin prior to blocking in goat serum. Very few CD4⁺ cells (2-3 per section, not shown) and CD8⁺ cells (2-3 per section, Figure 5.7a) were apparent.

Groups of mice were then irradiated twice a week for 3 weeks prior to FSA cell injection. Tumours were taken from these and unirradiated tumour-bearing control mice on days 9, 13 and 18 post FSA cell injection. Tumour sections were then stained for the presence of CD4⁺ and CD8⁺ cells. In this experiment however, no positive-staining cells could be seen on any tumour sections tested (results not shown).

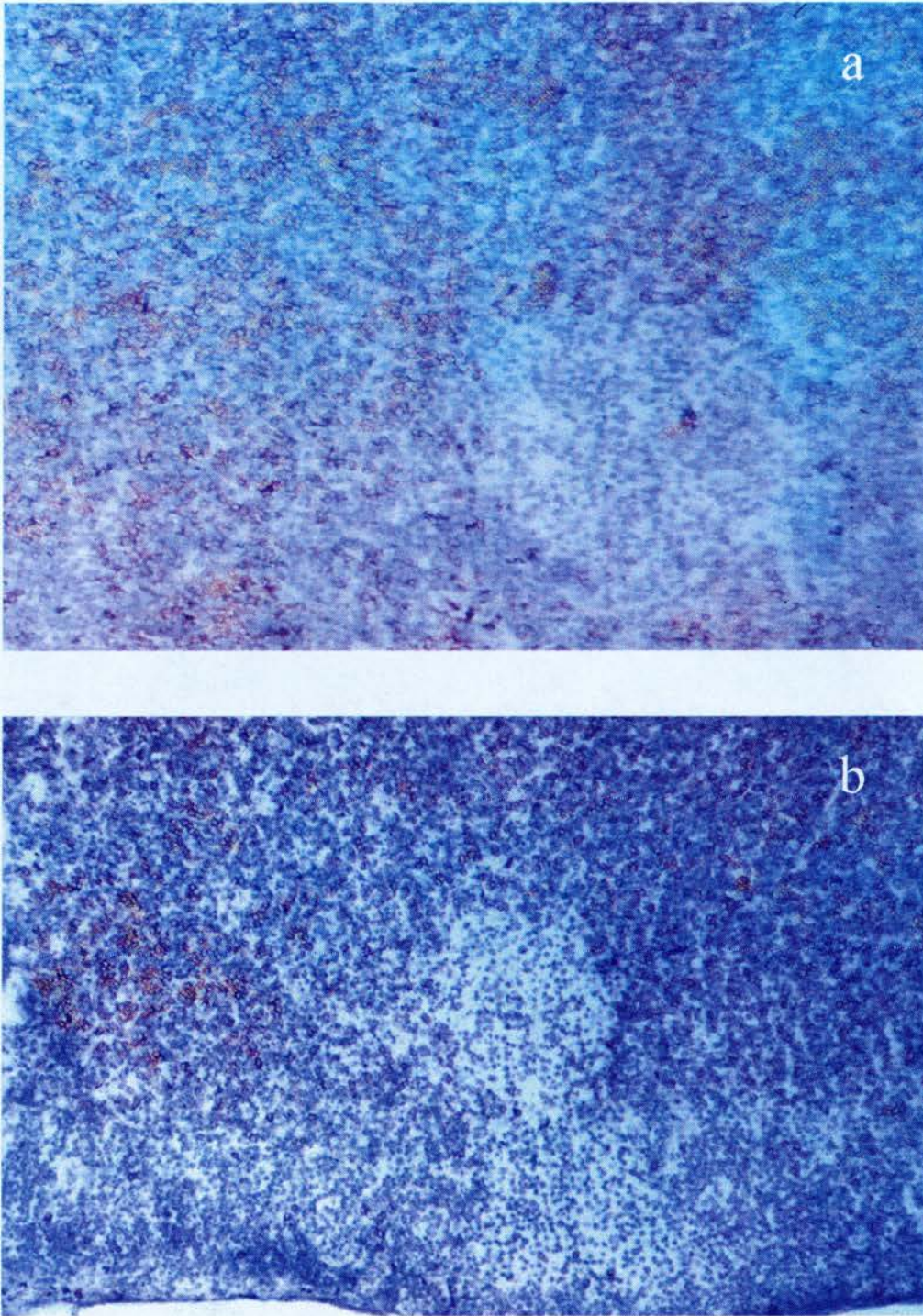


Figure 5.6. Immunohistochemical staining of LN sections. LN were taken from a normal mouse. Frozen sections were incubated overnight with (a) anti-CD4 antibody (1/2000 Dilution) or (b) anti-CD8 antibody (1/800 Dilution). A horseradish-peroxidase conjugated secondary antibody was then added, and positive cells (brown) were visualised using DAB. Sections were counterstained with haematoxylin, and show positive staining T cells in the parafollicular cortex surrounding a germinal centre.

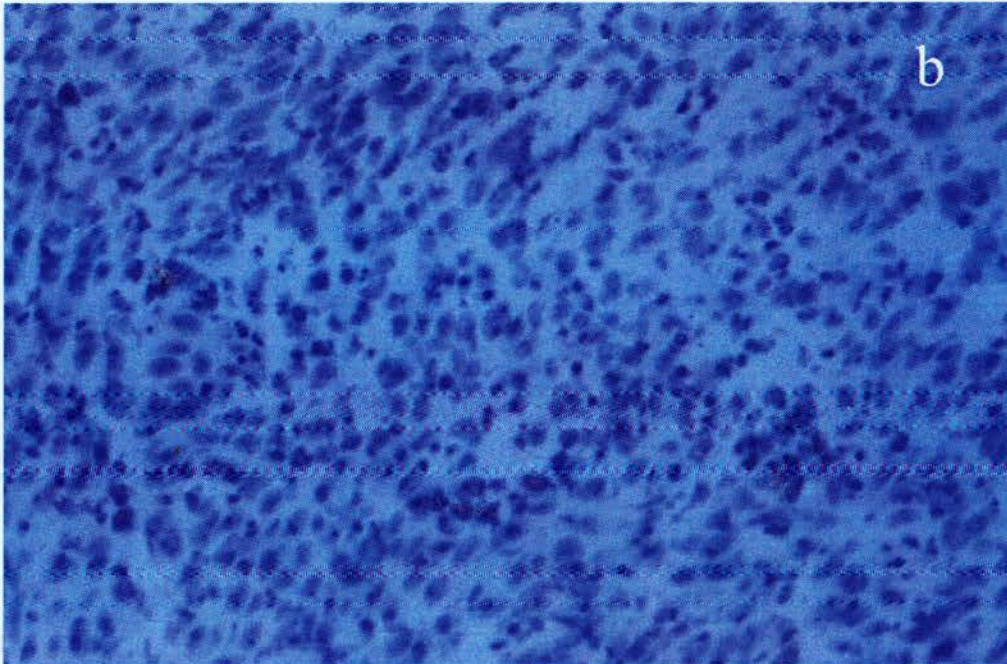
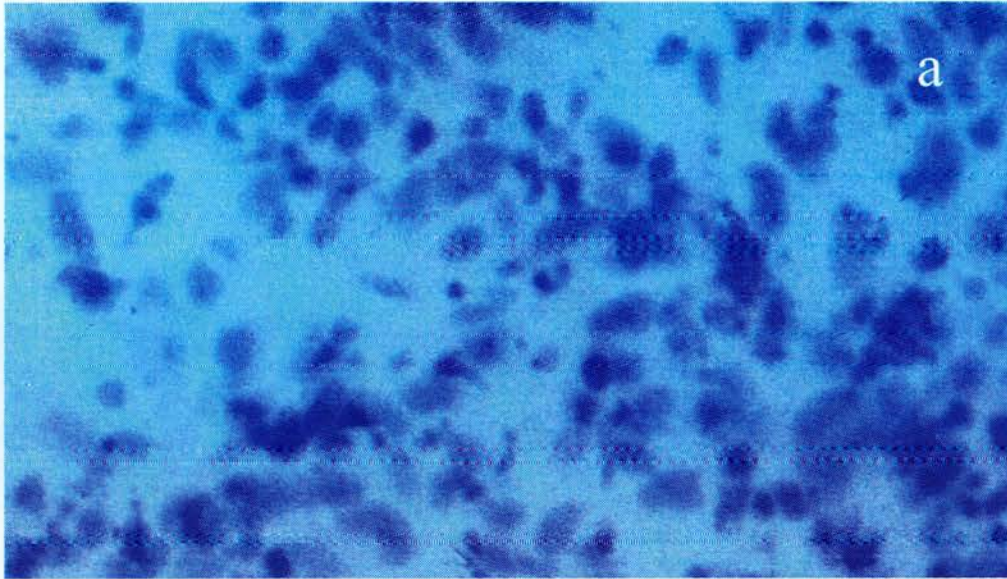


Figure 5.7. Immunohistochemical staining of FSA tumour sections. Tumours were taken from a normal mouse 22 days after FSA cell injection. Frozen sections were incubated overnight with (a) anti-CD8 antibody (1/800 Dilution) or (b) isotype control antibody. A horseradish-peroxidase conjugated secondary antibody was then added, and positive cells (brown) were visualised using DAB. Sections were counterstained with haematoxylin. a) shows a tumour-infiltrating cell staining positive for CD8 (far right of picture); b) shows non-specific background staining on a tumour section.

5.3 - Discussion

5.3.1 - Delayed-type hypersensitivity response to FSA cells

The FSA cells used in this study have previously been shown to be moderately immunogenic, in that they show increased growth in immunosuppressed mice, and decreased growth in mice that have been actively immunised with these cells (Suit and Kastellan, 1970). In the present study an attempt was made to develop an assay for anti-tumour responses in tumour-bearing mice, with the aim of investigating whether prior UVB exposure affects protective responses to the injected FSA cells, leading to the increased outgrowth of the cells in irradiated mice described in Chapter 4. A potential *in vivo* assay was a DTH response to the FSA cells. Immunocompetent mice received FSA cells either in 4 dorsal sites, leading to the formation of visible tumours, or intravenously, which results in the formation of colonies in the lung. Injection of killed FSA cells intrapinnally 14 days later failed to induce a DTH response in these mice.

Some previous studies investigating immune responses to tumours have demonstrated DTH responses to s1509a FSA cells (Grabbe *et al*, 1991; Campton *et al*, 2000). In the study by Grabbe *et al*, mice were immunised 3 times with tumour-antigen pulsed epidermal cells, prior to challenge with killed tumour cells. In the study by Campton *et al*, mice were immunised 3 times with killed cells prior to challenge with tumour-antigen pulsed dendritic cells. The lack of a DTH response in the present study could be due to the FSA cells in this study not sufficiently immunogenic to generate a measurable response at the elicitation stage of DTH. Alternatively, the nature of exposure of mice to the tumour antigens prior to the DTH assay may be important. Mice receiving skin grafts that had been incubated in tumour-extract were unable to mount a DTH response following challenge with whole tumour cells (Sluyter *et al*, 2001). In the

present study, mice received a single dose of live FSA cells prior to challenge with killed tumour cells; multiple exposure to tumour antigens prior to challenge may be required for a measurable DTH response. Additionally, the mice in the current study had developed tumours at the time of challenge, which may have affected the DTH response. Spleen cells from mice bearing tumours from FSA cells similar to those used in the present study contain a population of tumour-specific regulatory T cells (Howie and McBride, 1982); perhaps the presence of suppressor T cells prevented the induction of a DTH response in tumour-bearing mice. Using an immunisation protocol similar to those used in previous studies would have demonstrated whether the lack of a DTH response was due to the cells, or to the dose and number of injections of the FSA cells prior to the hypersensitivity assay.

5.3.2 - *In vitro* proliferation of LN cells in response to FSA cell antigens

In an attempt to develop an *in vitro* assay for specific anti-tumour responses in tumour bearing-mice, the *in vitro* proliferation of lymphoid cells from mice bearing FSA tumours in response to a preparation of FSA tumour antigens (TA) was tested. Cells from LN draining the site of FSA cell injection were cultured for 96 h in varying concentrations of a glycine extract of TA. DLN cells from mice that had been injected with FSA cells 20 days previously showed increased proliferation in response to TA, whereas the proliferation of DLN cells from naïve, tumour-free mice was not increased by the addition of TA. It appeared that the DLN of tumour-bearing mice contained tumour antigen-specific cells that responded to the TA preparation.

When this experiment was repeated to test the TA concentration that caused the largest increase in proliferation over a range of culture times, only a slight increase in

proliferation of DLN cells from tumour-bearing mice in response to TA was seen. A slight increase in proliferation of DLN cells from naïve controls in response to TA was also found; thus the small increase in proliferation of DLN cells did not appear to be due to tumour antigen-specific cells in the draining lymph node. A similar pattern was seen when spleen cells from tumour-bearing and control mice were cultured with TA.

The absence of an obvious tumour antigen-specific proliferative response by DLN cells from tumour-bearing mice could be due to the nature of the tumour extract used. The glycine extraction method was chosen because it has been used previously to produce viral antigen preparations from virus-infected cells that are more sensitive and effective than freeze/thaw preparations (Kettering *et al*, 1977). In the present study, a freeze/thaw preparation of TA was tested and found to have no effect on proliferation of DLN cells from tumour-bearing mice (results not shown).

The lack of DLN cell proliferation in response to the TA could result from the TA preparation being only weakly immunogenic; it is unlikely that these tumour cells have highly antigenic epitopes, as they are able to grow progressively in immunocompetent mice. It is possible that there were few tumour-antigen specific immune cells in the LN. Numerous previous studies have demonstrated the presence of tumour-specific cells in tumour DLN (Ellis *et al*, 1975; Yoshizawa *et al*, 1992); and tumour-specific cells have also been shown in the spleens of mice bearing tumours generated from FSA cells similar to those used in the present study (Howie and McBride, 1982). Alternatively, it may be that there were cells present in the LN that would have regulated the proliferation of any tumour antigen-specific cells in the present study; regulatory cells are found in the spleens of tumour bearing mice (Howie and McBride, 1982) and in the draining LN of melanoma patients (Hoon *et al*, 1987). Certainly no strong anti-tumour

responses could be detected in tumour-bearing mice using either an *in vivo* DTH or an *in vitro* proliferation assay in this study.

In Chapter 4, exposure of mice to broad-band UVB for a 3 week period prior to FSA cell injection resulted in increased tumour cell outgrowth in irradiated mice. This effect of UVB was a local one, such that increased growth only occurred if the cells were injected into the irradiated skin of UVB-exposed mice. In the current chapter, local immune parameters of irradiated and unirradiated tumour-bearing mice were compared with those of naïve, tumour-free mice. Mice were irradiated for 3 weeks and then received FSA cells in 4 dorsal sites; control groups received FSA cells without prior irradiation, were irradiated but not injected with FSA cells, or were untreated. Draining lymph nodes were taken on days 9, 13 and 18 post-FSA cell injection; the number of cells per lymph node were estimated, the spontaneous and mitogen-induced proliferation of the cells assessed, and the expression of cell surface markers by the DLN cells measured by flow cytometry. The experiment was repeated without the inclusion of the irradiated, tumour-free control group. During the second experiment, animals housed within the same room as the experimental animals were found to be infected with an intestinal parasite. Differences seen between the two experiments could have been partly due to infection of some of the mice in the second experiment.

5.3.3 - Cell numbers of LN draining the site of FSA cell injection

Draining lymph nodes taken from unirradiated tumour-bearing mice had approximately double the number of cells on day 9 post FSA cell injection, compared

with tumour-free mice. The number of DLN cells in tumour-bearing mice was decreased on day 13, but remained higher than the number of cells in the DLN of tumour-free mice throughout the experiment. This increase in LN cell numbers in tumour-bearing mice may have resulted either from expansion of cells within the lymph nodes, or recruitment of cells from other tissues, and is suggestive of an immune response in mice that have received FSA cells.

The cellularity of LN from irradiated, tumour-bearing mice was also increased compared with tumour-free mice; however, the DLN cell numbers of irradiated tumour-bearing mice did not increase to the same extent as that of unirradiated mice that had received FSA cells. The immune response to injected FSA cells in local LN may be decreased in irradiated mice compared to that of unirradiated mice.

In the second experiment, the number of cells in the LN of tumour-free mice was higher than the number in the first experiment. It is possible that parasitic infection of the mice increased the LN cell numbers in the second experiment; use of mice kept in a pathogen-free environment would have helped to avoid changes in the immune status of these mice.

5.3.4 - Spontaneous proliferation of DLN cells

The spontaneous proliferation of DLN cells taken from unirradiated tumour-bearing mice was higher than that of DLN cells from tumour-free mice on days 9, 13 and 18. Thus the increased cellularity of LN from tumour-bearing mice may be due to increased proliferation and therefore expansion of LN cell numbers. As an internal control, it might have been informative to assess whether there was any change in

proliferation of cells from LN not thought to be draining the tumours in tumour-bearing mice.

LN cells from tumour-bearing mice that had been irradiated prior to FSA cell injection generally had decreased spontaneous proliferation compared with LN cells from unirradiated tumour-bearing mice; this may be the reason that the increase in LN cell number following injection of FSA cells is less marked in UVB exposed mice. The decreased proliferation of LN cells from irradiated mice following FSA cell injection suggests that there is suppression of a response to the FSA cells in the UVB exposed mice. Other studies have demonstrated that mice irradiated prior to FSA cell injection develop regulatory cells that control the response to skin cancers in irradiated mice. Moodycliffe *et al* (2000) demonstrated that the spleens of mice exposed to UV for three times a week for 12-16 weeks contain natural killer T (NKT) cells that suppress tumour immunity when transferred to syngeneic recipients. Beissert *et al* (1999) demonstrated that alteration of the Th1/Th2 balance in favour of Th1 responses in mice, by disruption of the B7/CD23-CTLA-4 signalling pathway during photocarcinogenesis, reduced the number of skin tumours that developed. In the present study, it is possible that the development of regulatory T cells, and/or an alteration in Th1/Th2 responses in irradiated mice may have resulted in suppression of the immune response to the FSA cells.

It is possible that the increased proliferation of LN cells taken from tumour-bearing mice was a response to an antigen in the growth medium, rather than a reflection of *in vivo* proliferation of LN cells in response to the FSA cells. The FSA cells were washed prior to injection into the mice; however the mice may have received a small amount of a component of the growth medium, such as FCS. Previous studies

have demonstrated that FCS components can stimulate immune responses in mice (Slyuter *et al*, 2001; Porgador *et al*, 1996)). To avoid this, the use of normal mouse serum in place of FCS in the medium for proliferations was assessed; however, all concentrations (0.1% - 10%) of normal mouse serum tested were found to be toxic to the cells incubated alone or with Con A (results not shown). The increased cellularity of LN from tumour-bearing mice suggests that there is *in vivo* proliferation of the DLN cells, therefore the increased *in vitro* proliferation is more likely due to this than a reaction to the growth medium.

5.3.5 - Mitogen-stimulated proliferation of DLN cells

The proliferation of LN cells from tumour-bearing mice in response to two different mitogens, Con A) and PHA was generally higher than that of LN cells from tumour-free mice. The unstimulated proliferation of LN cells from tumour-bearing mice was also increased compared with LN cells from tumour-free mice; the amount of mitogen-stimulated proliferation mostly reflected the level of unstimulated proliferation.

Studies in human cancer patients have demonstrated altered mitogenic responses of leucocytes in these patients. Peripheral blood mononuclear cells (PBMC) from breast cancer patients had significantly decreased PHA-induced proliferation compared with PBMC from healthy controls (Witschke *et al*, 1995); the degree to which the proliferation decreased was dependent upon the tumour burden of these patients. In another study, PBMC from the majority of patients with solid tumours (including breast cancer, melanoma and basal cell carcinoma) responded normally to PHA or Con A. PBMC from only two patients (with oesophageal carcinoma) showed decreased proliferative responses (Miescher *et al*, 1986); however, tumour infiltrating lymphocytes

taken from all the patients did not proliferate or showed significantly lower responses to PHA and Con A. The stimulation index for the Con A response of LN from unirradiated tumour bearing mice was approximately half the stimulation index for that of unirradiated tumour-free mice at all time points. This may indicate a suppression of the Con A response in tumour-bearing mice; however, it may simply be a consequence of the higher background proliferation of LN cells taken from tumour-bearing mice.

In a study of melanoma patients, Hoon *et al* (1987) showed that cells from LN that are near to a melanoma have a higher Con A-induced proliferation than cells from LN further away. However, when the Con A expanded cells from LN close to melanomas were inactivated and incubated with autologous peripheral blood lymphocytes, these LN cells suppressed the PHA-induced stimulation of the blood lymphocytes to a greater extent than cells from LN further away from the melanoma. The LN cells taken from tumour-bearing mice in the present study that proliferated in response to Con A may have been a mix of effector and suppressor type cells. It would have been interesting to use the technique of Hoon *et al* (1987) to determine whether there was increased suppressive activity by LN cells from irradiated tumour-bearing mice, compared with unirradiated tumour-bearing mice.

5.3.6 - IL-10 production *in vitro* by DLN cells

Lymph node cells from both unirradiated and irradiated tumour-bearing mice produced higher amounts of IL-10 following 24, 48 and 96 h culture compared with LN cells from naïve, tumour-free controls. The amount of IL-10 detected in the culture supernatant was slightly higher for LN cells from unirradiated tumour-bearing mice after

24 and 48 h culture; however, after 96 h culture, more IL-10 was present in the culture supernatant for LN cells from the irradiated mice.

The increased production of IL-10 by LN cells from tumour-bearing mice may have been due to increased proliferation of LN cells from these mice, leading to increased IL-10 release. Regulatory cells are found in the spleens of mice with FSA tumours (Howie and McBride, 1982); it is possible that there are also regulatory cells in the LN of these mice, which might be the source of the IL-10 in the supernatants of cultures of LN cells from tumour-bearing mice.

IL-10 is present in the sera of patients suffering from different types of cancer (De Vita *et al*, 2000) and IL-10 transfected murine melanoma cells demonstrate increased tumour outgrowth compared with non-transfected cells (Garcia-Hernandez *et al*, 2002). IL-10 production by LN cells from tumour-bearing mice in the present study may help to down-regulate anti-tumour responses in these mice. IL-10 has been shown to reduce antigen-specific T cell proliferation *in vitro* (de Waal Malefyt *et al*, 1991); production of IL-10 by LN cells from tumour-bearing mice may have contributed to the lack of an anti-tumour antigen proliferative response of the LN cells described in section 5.2.2.

IL-10 is thought to be one of the mediators of UVB-induced immunosuppression; it is found in the skin of irradiated mice and is produced by murine keratinocytes after *in vitro* UVB exposure (Enk and Katz, 1992). LN cells from irradiated tumour-bearing mice showed increased IL-10 production compared with LN cells from unirradiated tumour-bearing mice after 96 h culture. Perhaps the increased IL-10 production by LN cells from irradiated tumour-bearing mice was due to an increased number of regulatory type or Th2 cells in the LN of these mice.

It may be that IL-10 production was an effect of culturing the cells *in vitro*; measurement of IL-10 in the serum of these mice would perhaps have given more information on the role of this cytokine in tumour outgrowth. Also, measurement of other cytokines such as IFN- γ , IL-12 and IL-4 would have established any differences in the cytokine profiles of irradiated and unirradiated tumour-bearing mice, and indicated whether a shift towards a Th2-type response occurred in irradiated mice.

5.3.7 - Phenotyping of DLN cells

In general, DLN cells from tumour-bearing mice had decreased numbers of cells expressing the cell surface markers Thy 1.2 and CD4 compared with cells from naïve controls. There was no consistent change in CD8 expression over the course of the two experiments. The significance of this decrease is not clear. It may be that CD4⁺ T cells (which also express the pan T cell marker Thy 1.2) are recruited from the lymph nodes to the site of FSA cell injection as part of the anti-tumour immune response.

LN cells expressing MHC class II from unirradiated tumour-bearing mice were increased at all time points tested, compared with unirradiated tumour-free mice. This increase may have been due to migration of MHC class II antigen presenting cells from the site of tumour cell injection to the LN, or recruitment from another site; alternatively it could have resulted from an increase in MHC class II expression by resident LN cells. In a guinea pig hepatocarcinoma model, administration of bacillus Calmette-Guerin into the tumour lesions resulted in tumour regression; this was accompanied by an increase in the number and MHC class II expression of draining LN cells (Steerenberg *et al*, 1991). Two-colour flow cytometry demonstrated that the increase in MHC class II expression was due to an increase in the number of T cells expressing MHC class II. In

the present study, injection of the FSA cells alone appears to have been sufficient to cause an increase in cellularity and MHC class II expression in the tumour DLN, perhaps due to their moderate immunogenicity. Use of two colour flow cytometry would have given more information as to the nature of the cells expressing MHC class II in this system.

MHC class II expression was also increased on LN cells from irradiated, tumour-bearing mice, compared with tumour-free controls at most time points measured; however, the increase was not as great as that of LN cells from unirradiated tumour-bearing mice. This suggests that the level of tumour-antigen presentation in irradiated mice may not be as high as that in unirradiated mice, perhaps resulting in decreased anti-tumour responses in irradiated mice, and increased tumour cell outgrowth.

5.3.8 - Detection of tumour-infiltrating cells

Enzymatic disaggregation of tumours taken from mice on days 18 and 24 post FSA cell injection, followed by flow cytometric analysis of the tumour cell suspensions, demonstrated the presence of CD11b⁺, Thy1.2⁺ and CD4⁺ infiltrating cells, indicating that the host cells within a tumour include macrophages and CD4⁺ T cells. This is in agreement with a previous assessment of cells infiltrating an FSA tumour (McBride *et al*, 1992). No CD8⁺ cells could be detected by flow cytometry; McBride *et al* (1992) demonstrated that approximately 3% of cells within an FSA tumour were CD8⁺, therefore it may be that in the present study there were too few cells present to detect. In an immunohistochemical study of cells infiltrating a murine melanoma, it was found that in mice irradiated for 3 weeks prior to melanoma cell injection, the number of infiltrating CD8⁺ cells was decreased compared with unirradiated controls (Donawho *et*

al, 1996). Therefore, in the present study, an immunohistochemical technique was used in an attempt to determine whether CD8⁺ cell numbers were altered in the present model.

Sections of a tumour taken from a normal mouse 22 days post FSA cell injection had very few CD4⁺ and CD8⁺ cells visible after immunoperoxidase staining. The majority of infiltrating cells in tumours induced by these FSA cells are macrophages, with a few CD4⁺ and CD8⁺ cells present (McBride *et al*, 1992). Many tumours exhibit infiltrating CD4⁺ and CD8⁺ cells although some studies have shown that they are functionally impaired (Van der Hove *et al*, 1997; Zhang *et al*, 1998).

In the present study, an attempt was made to compare the numbers of CD4⁺ and CD8⁺ cells in tumours taken from mice exposed to UVB over a period of 3 weeks prior to FSA cell injection with those taken from unirradiated mice. In this experiment however, the FSA cells used had been passaged once in an immunocompetent mouse, cultured *in vitro*, then injected into the experimental mice. No visible staining of CD4⁺ or CD8⁺ cells could be seen in tumours taken during this experiment. This could be due to the fact that the tumours were taken earlier than 22 days; however it is likely that FSA cells, which have already grown successfully in an immunocompetent mouse, are perhaps less immunogenic than the original FSA cells. Unfortunately, this experiment could not be repeated with the original cells due to time constraints.

5.4 - Summary of Chapter 5

Mice injected with live FSA cells failed to mount a measurable DTH response to killed FSA cells injected 14 days later. Tumour DLN cells taken from tumour-bearing mice and cultured *in vitro* in the presence of FSA TA did not show antigen-specific

proliferation. Lack of overt immune responses in tumour-bearing mice may be due to the presence of regulatory cells which down regulate anti-tumour responses in these mice.

LN cellularity was increased in tumour-bearing mice compared with tumour-free mice; *in vitro* spontaneous proliferation of LN cells from tumour-bearing mice was also increased compared with that of LN cells from tumour-free mice. In mice that had been exposed to UVB over a 3 week period prior to FSA cell injection, the number and spontaneous proliferation of LN cells were also increased compared with tumour-free mice; however, the increase in number or proliferation was not as high as that of LN cells from unirradiated tumour-bearing mice. Proliferation of LN cells from tumour-bearing mice in response to the mitogens Con A and PHA was generally increased, probably reflecting the increased background proliferation of these cells.

IL-10 production was higher for LN cells from tumour-bearing mice compared with LN cells from tumour-free mice, possibly due to the presence of regulatory cells, or a Th2 cells, in the LN of tumour-bearing mice.

The number of cells expressing CD4 and Thy 1.2 in LN taken from unirradiated tumour-bearing mice at days 9, 13 and 18 post-FSA cell injection was decreased compared with tumour-free mice. LN cells taken from irradiated tumour-bearing mice also showed a decrease in CD4 and CD8 expression, although to a lesser extent than LN cells from unirradiated tumour-bearing mice. MHC class II expression by LN cells from unirradiated tumour-bearing mice was increased compared with LN cells from tumour-free mice; again the difference in MHC class II expression by LN cells from irradiated tumour-bearing mice was less marked than that of LN cells from unirradiated tumour-bearing mice.

Taken together, these results are indicative of an immune response in tumour-bearing mice, which may be regulated by the presence of regulatory cells in these mice. Changes in immune parameters following FSA cell injection are less marked in mice that have been exposed to UVB compared with unirradiated mice; it is possible that UVB exposure results in an environment that allows increased numbers of regulatory cells to be generated locally in these mice, resulting in enhanced tumour outgrowth.

Much of the work presented in this chapter was of a preliminary nature. Should this work be continued in the future, I would recommend that the cytokine profiles of the T cells from the DLN be assessed, to determine whether different subsets of T cells are found in unirradiated and irradiated mice. I would also suggest that the proliferating DLN cells from tumour-bearing mice be identified, to improve the understanding of the importance of different immune cells in anti-tumour immune responses.

Chapter 6

The effects of urocanic acid on FSA cell outgrowth *in vivo* and lymphocyte proliferation *in vitro*

6.1 - Introduction

Exposure of skin to UVB causes photoisomerisation of *trans*-urocanic acid (UCA), present in the stratum corneum, to *cis*-UCA (Anglin *et al*, 1961). Numerous studies have demonstrated *cis*-UCA to be one of the mediators of UV-induced immunosuppression. For example, tape stripping of mice to remove the stratum corneum prior to UV exposure abrogated UV-induced suppression of CH (De Fabo and Noonan, 1983). Treatment of mice with *cis*-UCA mimicked some of the suppressive effects of UV on DTH and CH responses (Ross *et al*, 1986; Kurimoto and Streilein, 1992), and delayed rejection of skin and other allografts (Gruner *et al*, 1992a; Gruner *et al* 1992b). Finally, injection of mice with a monoclonal antibody specific for *cis*-UCA prior to UVB exposure completely restored the DTH response to alloantigen in irradiated mice (Moodycliffe *et al*, 1996).

Two previous studies have suggested a role for UCA in photocarcinogenesis. Reeve *et al* (1989) demonstrated that topical application of UCA prior to irradiation of mice with solar-simulating UV during UV carcinogenesis increased both the number and malignancy of the resultant tumours, compared with mice receiving only UV. More recently, Beissert *et al* (1997) used tumour antigen-pulsed epidermal cells to immunise mice against a S1905 spindle cell tumour. Mice that had received epidermal cells incubated in *cis*-UCA prior to tumour antigen pulsing, were unable to reject subsequently injected tumour cells; these mice also showed a significantly suppressed

DTH response to the tumour cells. In the current chapter, the effect of *in vivo cis*-UCA treatment on the outgrowth of subsequently injected FSA cells is assessed. Additionally, a monoclonal antibody specific for *cis*-UCA is used to determine whether *cis*-UCA is a critical mediator in the UVB-enhanced FSA cell outgrowth described in Chapter 4.

The mechanism for induction of suppression by *cis*-UCA remains to be elucidated; however, numerous potential pathways have been suggested, including degranulation of mast cells and histamine release (Wille *et al*, 1999), effects on APC (Noonan *et al*, 1988) and production of prostaglandin by epidermal cells (Hart *et al*, 1993). A study by Holan *et al* (1998) demonstrated that *cis*-UCA could act directly on T cells *in vitro* to suppress proliferation, and enhance release of IL-10. An attempt is made in the current chapter to reproduce some of the approaches of Holan *et al*.

6.2 - Results

6.2.1 - Effect of *in vivo* treatment with *cis*-UCA on FSA cell outgrowth

Treatment of mice topically with a single dose of 0.1 µg -200 µg of *cis*-UCA prior to infection of the mice with HSV suppresses the DTH response to the virus (Ross *et al*, 1986; Norval *et al*, 1989). In the present study, topical application of 100 µg *cis*-UCA onto shaved dorsal skin twice a week for 3 weeks prior to FSA cell injection (section 2.7.1) had no effect on the outgrowth of FSA cells compared with controls (Figure 6.1a). This experiment was repeated with similar results. Continuing topical *cis*-UCA application after FSA cell injection also had no effect on FSA cell outgrowth (Figure 6.1a). To exclude the possibility that this was due to a lack of uptake of *cis*-UCA through the skin, *cis*-UCA was injected intradermally twice a week for 3 weeks (section

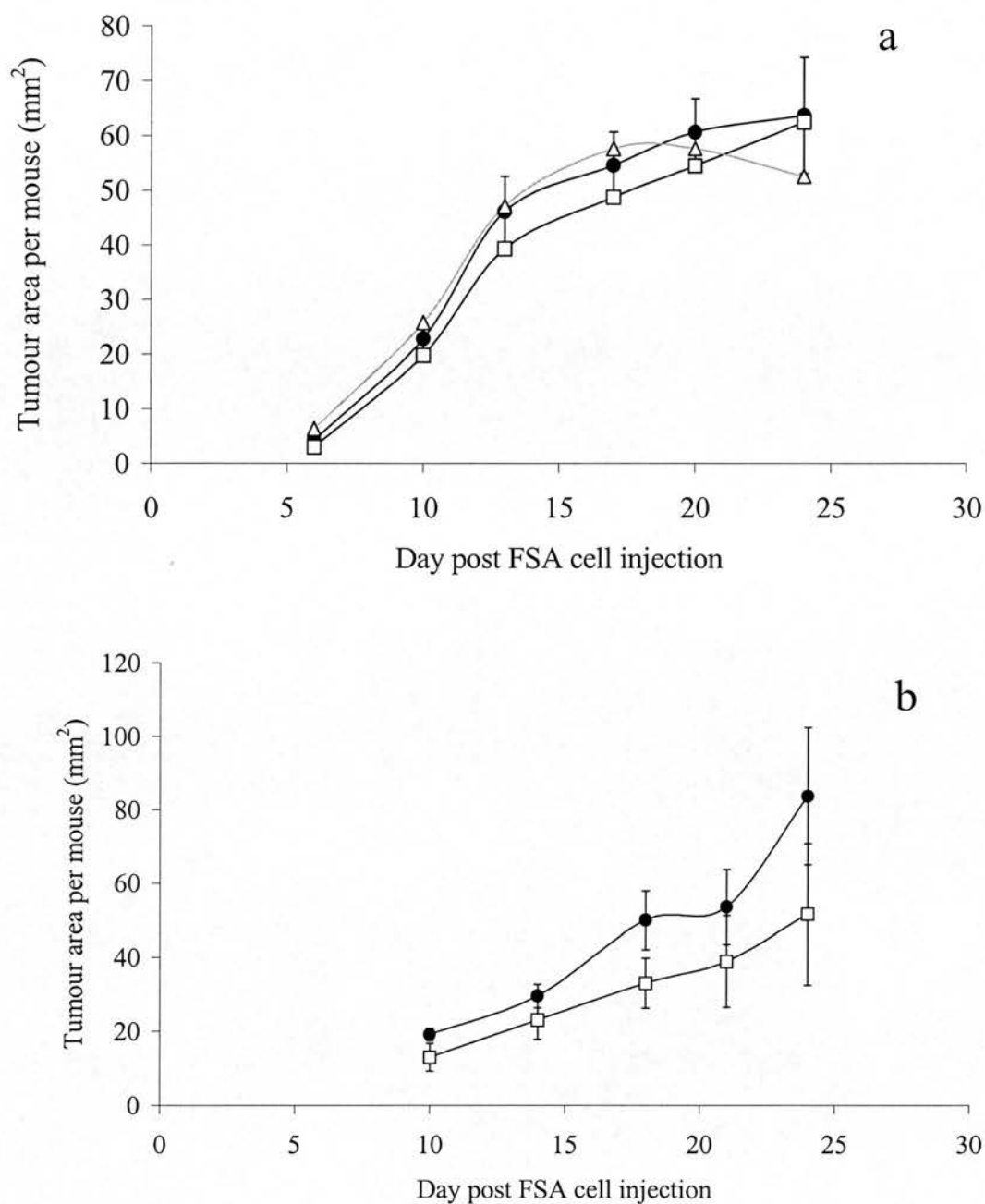


Figure 6.1. Growth of FSA cells following 3 weeks *cis*-UCA treatment. a) Groups of shaved mice (n=10) were treated twice a week for 3 weeks with 100 μ g *cis*-UCA topically (□). Control mice were not treated (●). FSA cells were injected into the treated skin 24 h after the final *cis*-UCA application. One group continued to receive 100 μ g *cis*-UCA topically twice a week after FSA cell injection (Δ); b) Groups of shaved mice (10-17) were injected intradermally twice a week for 3 weeks with 100 μ g *cis*-UCA (□). Control mice were not treated (●). FSA cells were injected into the treated skin 24 h after the final *cis*-UCA application. There was no significant difference between the *cis*-UCA and control groups at any time point.

2.7.2); there was no significant difference in tumour size between these mice and controls (Figure 6.1b).

6.2.2 - Effect of treatment with *cis*-UCA on formation of tumours in the lung

To assess whether *cis*-UCA acted systemically to affect the formation of tumours in the lung, mice were treated topically twice a week for 3 weeks with 100 µg *cis*-UCA or 100 µg *trans*-UCA. FSA cells (7.5×10^4 per mouse) were injected into the tail vein 24 h after the treatment; two weeks later, mice were killed, their lungs removed and the number of lung colonies counted after fixation (described in section 2.4.3). Table 6.1 shows the results of three separate experiments. The number of colonies produced in the lungs varied greatly between mice within a single group. No consistent effect of *cis*-UCA treatment was seen on the number of lung colonies formed.

Experiment	Mean number of lung colonies per mouse (range)		
	Control	<i>Cis</i> -UCA	<i>Trans</i> -UCA
1	11 (1-31)	39 (17-65)	ND
2	36 (7-108)	42 (6-76)	57 (3-159)
3	41 (6-168)	11 (3-24)	ND

Table 6.1. Effect of *cis*-UCA treatment on formation of lung colonies by FSA cells. Groups of mice (9-15) were treated topically twice a week for 3 weeks with 100 µg *cis*- or *trans*-UCA. FSA cells were injected intravenously 24 h after the final UCA treatment. The lungs were removed 14 days later and stained using Bouin's fluid. Numbers in brackets show the range. ND: not done.

In experiments 1 and 2, the number of lung colonies was increased in *cis*-UCA-treated mice; in experiment 3, the number of lung colonies was decreased in *cis*-UCA treated mice. In one experiment, the effect of *trans*-UCA treatment on formation of lung tumours was assessed (Table 6.1). The number of lung colonies was increased in this group compared with control mice; however this was not significant.

6.2.3 - Effect of *cis*-UCA-specific antibody on UVB-enhanced FSA cell outgrowth

Injection of mice with 0.9 µg of a monoclonal antibody specific for *cis*-UCA prior to a single UV exposure abrogated UV-induced suppression of DTH to alloantigen (Moodycliffe *et al*, 1996) and to HSV (El-Ghorr and Norval, 1995), and partially reversed UV-induced suppression of CH to DNFB (Kondo *et al*, 1995). To assess the role of *cis*-UCA in UVB-enhanced tumour cell outgrowth in the present study, mice were irradiated twice a week for 3 weeks prior to FSA cell injection (as described in section 2.1.4); *cis*-UCA antibody was injected intraperitoneally 2 h before each irradiation (section 2.7.3). Injection of 0.5 µg *cis*-UCA antibody prior to each exposure with 1200 J/m² broad-band UVB slightly decreased tumour outgrowth in irradiated mice, compared with UVB-exposed mice treated with control antibody; however this difference was not significant (Figure 6.2).

Increasing the dose of *cis*-UCA antibody to 5 µg protein per mouse also had no significant effect on tumour outgrowth in irradiated mice (results not shown). Injection of *cis*-UCA antibody had no effect on the outgrowth of tumours in unirradiated mice (Figure 6.2).

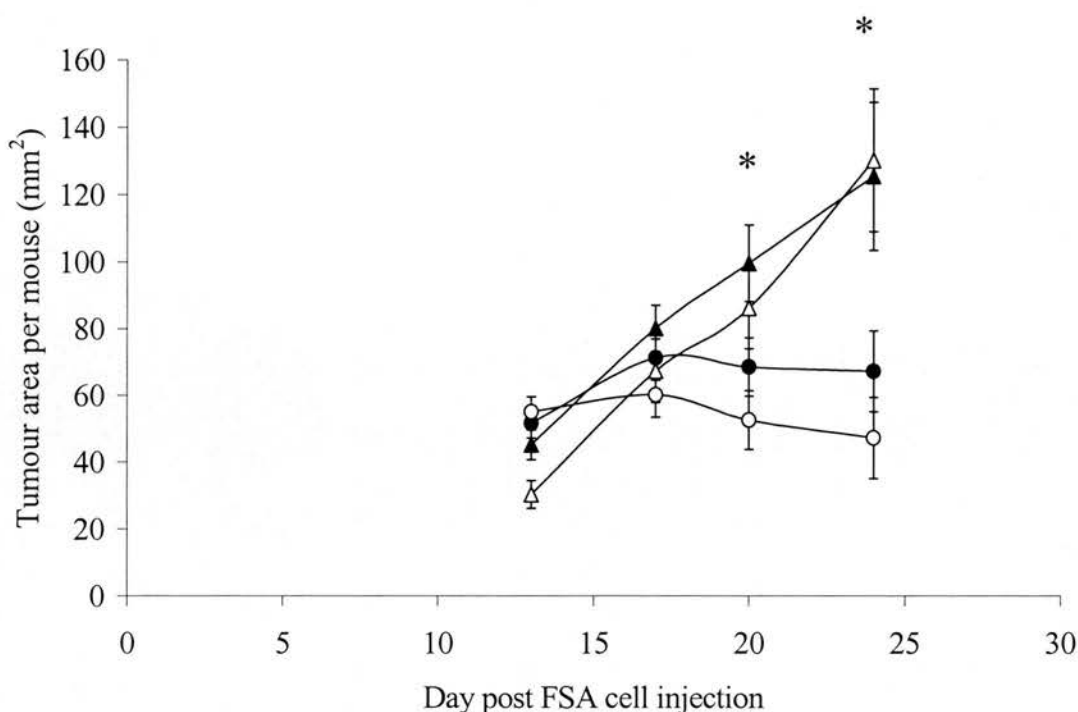


Figure 6.2. Effect of *cis*-UCA specific antibody on UVB-enhanced FSA cell outgrowth.

Groups of 14 mice were irradiated twice a week for 3 weeks with 1200 J/m² broad-band UVB; two hours before each irradiation mice were injected i.p. with 0.5 µg *cis*-UCA antibody (Δ) or an isotype control antibody (▲). Control mice received *cis*-UCA antibody (○) or isotype control antibody (●) but were not irradiated. * shows a significant difference ($p < 0.05$) between the unirradiated groups and the irradiated groups.

6.2.4 - Effect of UCA isomers on *in vitro* proliferation of spleen cells

Holan *et al* (1998) demonstrated that pre-incubation of spleen cells from BALB/c mice for 24 h in *cis*-, but not *trans*-UCA, suppressed their ability to proliferate in response to stimulation with anti-CD3 antibody. In the present study, the effect of UCA isomers on anti-CD3 induced proliferation of spleen cells from C3H/HeN mice was assessed, as described in Section 2.7.4. Initially, the effect of pre-incubation of spleen cells with 200 µg/ml *cis*- or *trans*-UCA for 2 or 24 h prior to stimulation with anti-CD3 was tested. Figure 6.3 shows that incubation of spleen cells in *cis*-UCA for 2 or 24 h

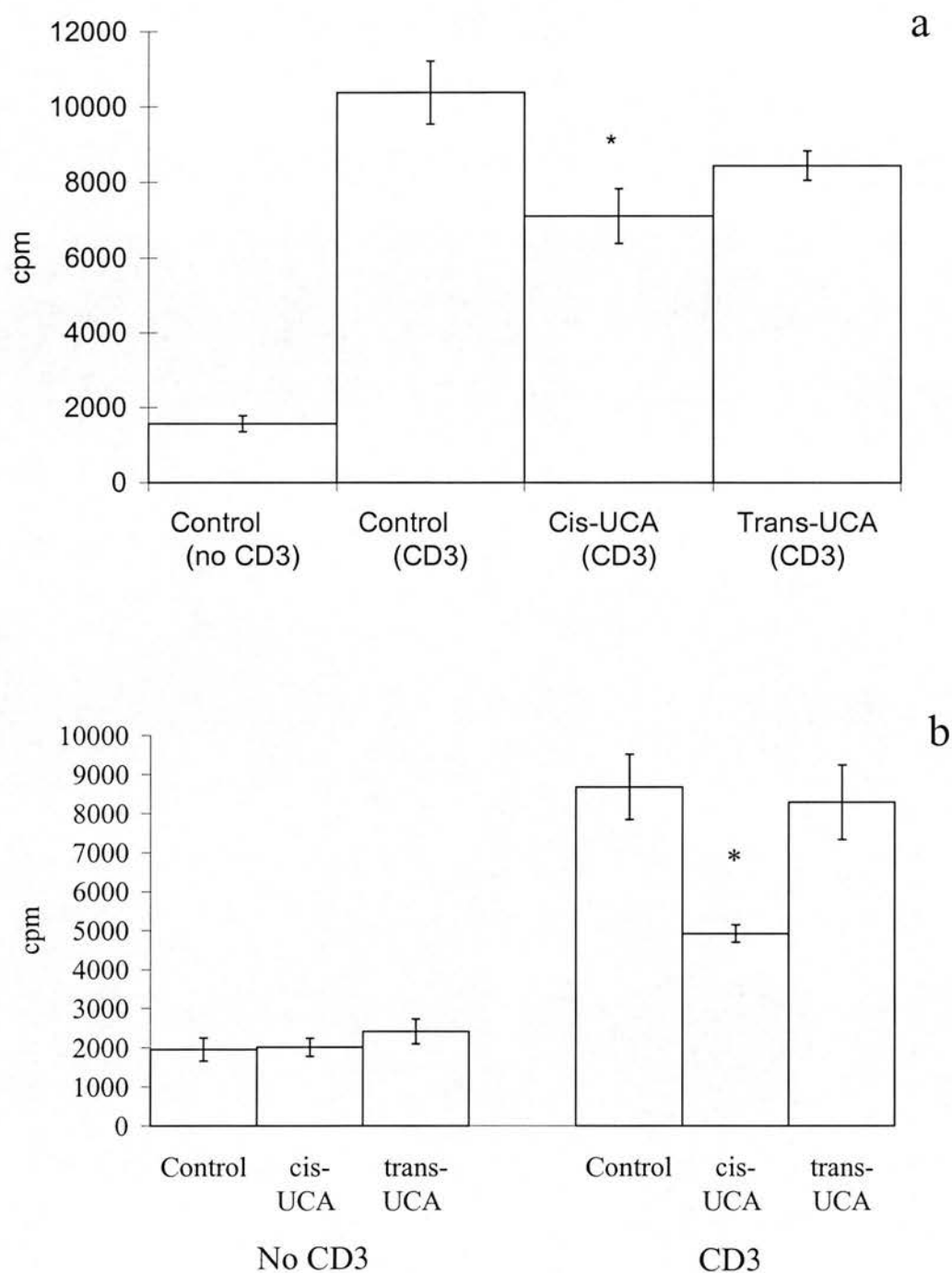


Figure 6.3. The effect of incubation time on *cis*-UCA induced suppression of spleen cell proliferation in response to CD3 antibody. Spleen cells from C3H/HeN mice were incubated in medium alone, or in 200 μ g/ml *cis*- or *trans*-UCA for (a) 2 h or (b) 24 h. Cells were then washed twice and incubated for 72 h with or without 2 μ g/ml CD3 antibody; 3 H-Thymidine was added for the final 8 h of culture. The average cpm for 6-7 replicate wells is shown; error bars show the SEM. * indicates a significant decrease ($p < 0.05$) in proliferation of UCA treated cells compared with control cells.

caused a significant decrease in the proliferative response of these cells, compared with controls; the suppression was much greater in cells incubated for 24 h than 2 h in *cis*-UCA. Incubation of the cells in *trans*-UCA however, had no effect on their proliferation in response to anti-CD3 (Figure 6.3). The 24 h hour pre-incubation in UCA of spleen cells followed by anti-CD3 induced proliferation was repeated on 4 separate occasions (Table 6.2). *Cis*-UCA incubation suppressed proliferation in 3 out of 4 experiments; however incubation in *trans*-UCA also resulted in a decreased proliferative response in 2 out 4 experiments, although not to the same extent as *cis*-UCA incubation. In 3 out of 4 experiments, *cis*-UCA pre-incubation slightly suppressed the background level of unstimulated spleen cells.

Experiment	No CD3			CD3		
	Control	<i>Cis</i> -UCA	<i>Trans</i> -UCA	Control	<i>Cis</i> -UCA	<i>Trans</i> -UCA
1	1559 (81)	1773 (109)	1814 (81)	11676 (803)	6842 (552) †	7148 (392) †
2	2521 (107)	1939 (94) †	2356 (243)	5620 (418)	4651 (488)	4972 (495)
3	3654 (159)	2484 (247) †	3962 (171) *	7051 (678)	4923 (316) †	7149 (264) *
4	2626 (193)	1682 (205) †	2685 (306) *	10827 (440)	3309 (481) †	6249 (482) † *

Table 6.2. Effect of UCA isomers on in vitro proliferation of spleen cells in response to CD3 antibody. Spleen cells from C3H/HeN mice were incubated alone, or in 200 µg/ml *cis*- or *trans*-UCA for 24 h. Cells were then washed twice and incubated for 72 h with or without 2 µg/ml CD3 antibody; ³H-Thymidine was added for the final 8 h of culture. Numbers are expressed as the mean cpm (SEM) of 4-8 replicate wells. † indicates a significant decrease (p<0.05) in proliferation of UCA treated cells compared with control cells. * indicates a significantly higher (p<0.05) proliferation of *trans*-UCA treated cells compared with *cis*-UCA treated cells.

The effect of pre-incubation in a lower concentration of UCA and in a mixture of *cis*- and *trans*-UCA on proliferation of spleen cells was also tested. The results of a representative experiment are shown in Figure 6.4. Incubation in 100 µg/ml *cis*- or *trans*-UCA for 24 h prior to stimulation with anti-CD3 had no effect on the proliferative response; however, pre-incubation in a mixture of 100 µg/ml *cis*- and 100 µg/ml *trans*-UCA significantly suppressed the anti-CD3 induced proliferation of spleen cells. Pre-incubation in the two UCA isomers had no effect on the unstimulated proliferation of the spleen cells in this experiment (Figure 6.4).

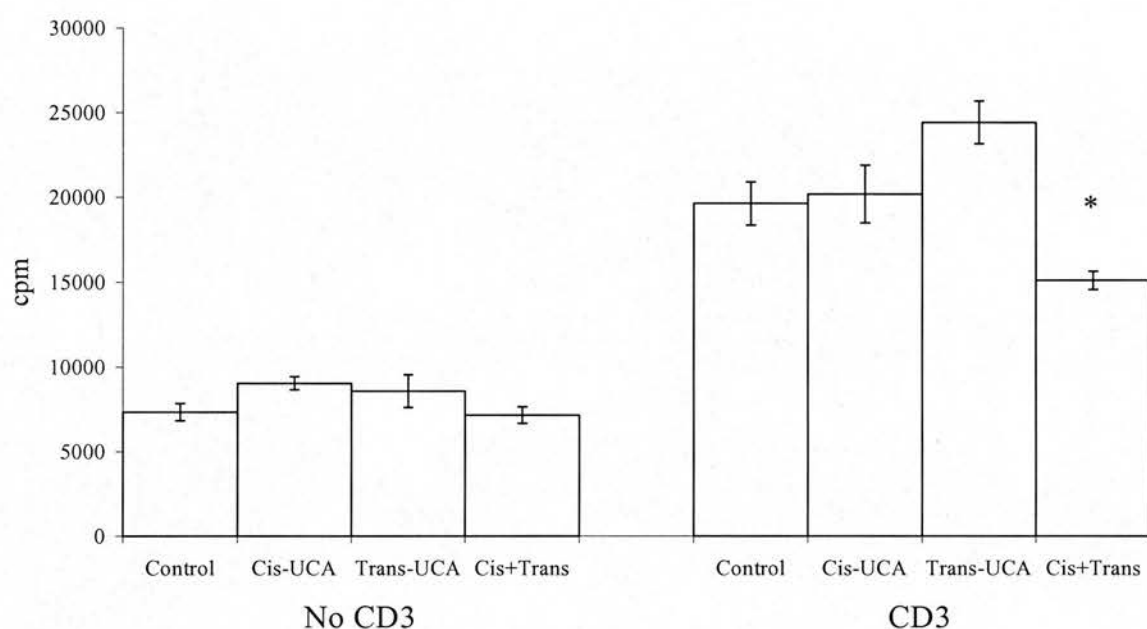


Figure 6.4. Effect of combining *cis*-UCA and *trans*-UCA on proliferation of spleen cells in response to CD3 antibody. Spleen cells from C3H/HeN mice were incubated in medium alone, or in 100 µg/ml *cis*- or *trans*-UCA, or in 100 µg/ml *cis*- UCA + 100 µg/ml *trans*-UCA. Cells were then washed twice and incubated for 72 h with or without 2 µg/ml CD3 antibody; ³H-Thymidine was added for the final 8 h of culture. The average cpm for 4-5 replicate wells is shown; error bars show the SEM. The experiment was repeated with similar results. * indicates a significant decrease ($p < 0.05$) in proliferation of UCA treated cells compared with control cells.

Holan *et al* (1998) demonstrated that incubation of BALB/c spleen cells in *cis*-UCA during anti-CD3 stimulation induced IL-10 mRNA expression, that was not seen in cultures incubated alone or with *trans*-UCA. In the present study, spleen cells from C3H mice were stimulated for 20 h with anti-CD3 in the presence or absence of *cis*- or *trans*-UCA, as described in section 2.7.4. The total RNA was then extracted, and used to produce cDNA; this was amplified by PCR in the presence of primers for IL-10, or the house-keeping gene β -actin, (sections 2.7.5-2.7.8). As seen in Figure 6.5, IL-10 mRNA was expressed to a similar level in unstimulated and CD3-stimulated cells, regardless of whether they were incubated in the presence of *cis*- or *trans*-UCA.

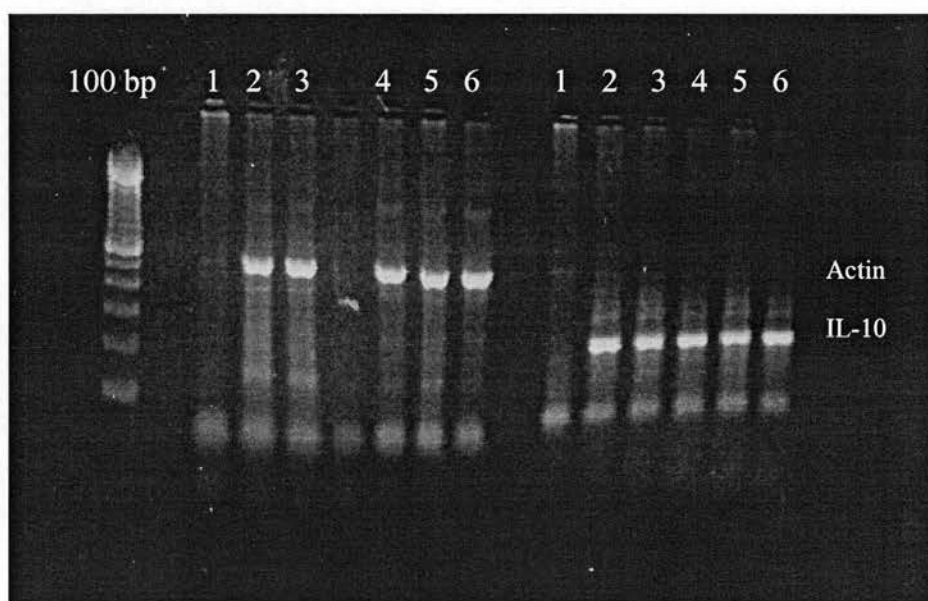


Figure 6.5. IL-10 mRNA expression by spleen cells stimulated in the presence of UCA. Spleen cells from C3H/HeN mice were incubated for 20 h alone (lane 2), or in 200 μ g/ml *cis*-UCA (lane 3); or were stimulated with anti-CD3 alone (lane 4) or in the presence of 200 μ g/ml *cis*-UCA (lane 5) or 200 μ g/ml *trans*-UCA (lane 6). Total RNA was isolated, reverse transcribed, and amplified by PCR with 5' and 3' primers for IL-10 and actin. PCR products were run on an agarose gel and visualised using ethidium bromide. Lane 1 shows RNA from anti-CD3-stimulated cells amplified by PCR with the same primers, but without prior reverse transcription to give cDNA. The intensity of the bands was measured and found to be similar for all samples. This experiment was repeated with similar results.

6.3 - Discussion

6.3.1 - Effect of *in vivo* treatment with *cis*-UCA on FSA cell outgrowth

The *cis*-isomer of UCA, formed upon irradiation of *trans*-UCA, mediates at least some of the suppressive effects of UV. Treatment of mice with *cis*-UCA has been shown to suppress DTH responses to HSV (Norval *et al*, 1989), and prolong the survival of skin allografts and prevent graft-versus-host disease (Gruner *et al*, 1992). Suppression of CH responses following *cis*-UCA treatment has also been demonstrated (Lauerma *et al*, 1995; Kurimoto and Streilein, 1992a).

In the present study, treatment of mice topically with *cis*-UCA for 3 weeks prior to FSA cell injection had no effect on tumour outgrowth, compared with untreated controls. It is possible that the lack of effect was due to an uptake problem; however, this is unlikely as a previous study has shown that radioactive *cis*-UCA applied to mouse skin is readily internalised (Hug and Hunter, 1991). In the present study, injection of *cis*-UCA intradermally prior to FSA cell injection also had no effect on tumour outgrowth. Continuation of topical *cis*-UCA treatment after FSA cell injection failed to affect tumour outgrowth. Therefore *cis*-UCA did not act directly on the tumour cells to alter growth.

The lack of an effect of *cis*-UCA on tumour outgrowth in this study was in contrast to the demonstration by Reeve *et al* (1989) that topical treatment of mice with UCA during UV carcinogenesis increased the number of resultant tumours. In that study, the UCA was applied prior to each UV exposure, therefore it is possible that products were formed from the photo-oxidation of UCA by UVB; these products are thought to have a role in UV-induced immune suppression (Kammeyer *et al*, 2001). Additionally, in the study by Reeve *et al* (1989), UCA treatment may have caused an increase in

induction of tumours, rather than promotion of tumour outgrowth by suppression of anti-tumour immune responses.

Beissert *et al* (1997) also demonstrated a role for *cis*-UCA in tumour outgrowth. EC that were pulsed with TA *in vitro* were used to immunise mice against tumour challenge *in vivo*; EC incubated in *cis*-, but not *trans*-UCA prior to TA pulsing failed to induce anti-tumour immunity upon injection into mice. This effect of *cis*-UCA could be abrogated by the addition of IL-12 to the EC during or after the *cis*-UCA incubation (Beissert *et al*, 2001). As seen in chapter 4, IL-12 treatment *in vivo* did not reverse UVB-enhanced tumour cell outgrowth in the current study. The conflicting results of the studies by Beissert *et al* with the present one could be due to the different protocols used; immunisation with tumour antigen-pulsed EC may well induce a stronger, or different, immune response compared with that resulting from the injection of dividing tumour cells. *In vitro* treatment of EC with *cis*-UCA is likely to affect the majority of cells; *in vivo* treatment of mice with *cis*-UCA may only affect some of the susceptible cells, such that there remain cells that are unaffected and able to induce an immune response in the usual way.

Immunosuppression following *cis*-UCA treatment has mostly been seen following a single dose of *cis*-UCA prior to sensitisation. One study looking at the effect of chronic treatment of mice with *cis*-UCA demonstrated that intradermal injection of *cis*-UCA three times a week for four weeks failed to suppress the DTH response to HSV or the CH response to oxazalone (El-Ghorr and Norval, 1997b). It was suggested by the authors that repeated exposure of mice to high doses of *cis*-UCA may result in adaptation, or lead to the occupation of putative *cis*-UCA receptors, such that they become unresponsive.

As seen in Chapter 4, exposure of mice to broad-band UVB, but not TL01, prior to FSA cell injection resulted in increased outgrowth of the tumour cells. During chronic irradiation of mice with doses of 1000 J/m² broad-band UVB, or 3000 J/m² TL01 three times each week, an increase in *cis*-UCA was seen after the first week, and a maximum of 38 % and 35 % *cis*-UCA was reached with the broad-band UVB and TL01 lamps respectively (El-Ghorr *et al*, 1995). Taking the results of that study and the present one together, there does not appear to be a correlation between UCA conversion and increased FSA cell outgrowth.

6.3.2 - Effect of treatment with *cis*-UCA on formation of tumours in the lung

Wavelengths within the UVB range do not penetrate past the upper layer of the dermis; therefore it is likely that UV-induced systemic suppression is initiated through a chromophore in the skin, such as *cis*-UCA. Subcutaneous injection of *cis*-UCA causes systemic suppression of CH responses (Hart *et al*, 1997). *Cis*-UCA is found in the serum of irradiated mice after UVB exposure (Moodycliffe *et al*, 1993) and in the urine after whole body UVB exposure of human volunteers (Kammeyer *et al*, 1997).

In the present study, an attempt was made to assess any systemic effect of *cis*-UCA on tumour outgrowth, by topical application of *cis*-UCA over a 3 week period prior to intravenous FSA cell injection. No consistent effect of *cis*-UCA on the number of lung tumours formed was apparent; however, the variability of the technique, as discussed in Chapter 4, section 4.3.6, may have masked any changes in the *cis*-UCA treated mice.

6.3.3 - Effect of *cis*-UCA-specific antibody on UVB-enhanced FSA cell outgrowth

Injection of a monoclonal antibody specific for *cis*-UCA prior to UVB irradiation of mice prevents UV-induced suppression of DTH to HSV or alloantigen, and UV-induced reduction in epidermal LC (Moodycliffe *et al*, 1996; El-Ghorr and Norval, 1995). In the present study, injection of anti-*cis*-UCA antibody prior to each irradiation did not reduce the enhanced outgrowth of FSA cells injected s.c after 3 weeks UVB exposure. It is possible that the dose of antibody received by the mice in the present study (0.5 µg) prior to each irradiation was not high enough to reverse the effects of UV, however a high dose of antibody (5 µg/mouse prior to each irradiation) was tested and found to be ineffective.

The results of this study are in contrast to the results of a study by Beissert *et al* (2001), where mice were irradiated over a period of 6 months, resulting in induction of skin tumours; one group received 0.1 µg *cis*-UCA specific antibody prior to each irradiation. In that study, the probability of tumour induction in irradiated mice receiving no antibody, or a control antibody was almost 100% at day 200 of the experiment; treatment of mice with anti-*cis*-UCA antibody reduced this probability to around 50%. The *cis*-UCA specific antibody was initially raised in a BALB/c mouse; therefore, it may be that multiple injections of the antibody into C3H mice in the present study resulted in the production of anti-idiotypic antibodies, which reduced the effectiveness of the anti-*cis*-UCA antibody. Beissert *et al* (2001) used BALB/c mice, which could explain why the antibody was effective in that study.

It may be that *cis*-UCA has role in the early stages of tumour induction, but does not have a role in UV-induced suppression of immune responses to injected tumour cells. Injection of anti-*cis*-UCA antibody does not reverse all the effects of UVB

irradiation. In single dose experiments, it had no effect on UV-induced suppression of CH, or DC accumulation in DLN (El-Ghorr and Norval, 1995); however, anti-*cis*-UCA did prevent the induction of transferable suppressor cells in irradiated mice (Moodycliffe *et al*, 1996). Thus *cis*-UCA is a mediator of only some aspects of UV-induced immunosuppression; the results of the present study do not show a role for *cis*-UCA in suppression of anti-tumour responses in irradiated mice, suggesting that other effects of UVB exposure such as DNA damage, cytokine release, and antigen presenting cell defects may be more important in UVB-enhanced tumour outgrowth.

6.3.4 - Effect of UCA isomers on *in vitro* proliferation of and IL-10 mRNA expression by spleen cells

The mechanism of action of *cis*-UCA that results in immunosuppression is not entirely clear. It has been suggested that *cis*-UCA suppresses CH responses through induction of TNF α (Kurimoto and Streilein, 1992), although this has been disputed (Moodycliffe *et al*, 1994). *Cis*-UCA may cause release of immunosuppressive cytokines from keratinocytes. However, two *in vitro* studies have failed to find an effect of *cis*-UCA on secretion/expression of a number of cytokines, including IL-10 and TNF α (Redondo *et al*, 1996; Zak-Prelich *et al*, 2001). *Cis*-UCA administration causes loss of LC from the epidermis (Norval *et al*, 1990), though it is not known whether this is a direct effect on the LC, or via an intermediate; Rattis *et al* (1995) failed to demonstrate an effect of *cis*-UCA on the allostimulatory function of human LC *in vitro*. In an *in vitro* study by Holan *et al* (1998), *cis*-UCA was shown to reduce proliferation of splenocytes from BALB/c mice, as a result of IL-10 production by CD4⁺ T cells; it was suggested

that the *cis*-UCA induced IL-10 may act on APC to affect the induction of an immune response.

In the present study, an attempt was made to reproduce some of the work of Holan *et al*, using splenocytes from C3H/HeN mice. Pre-incubation of spleen cells for 2 h in 200 µg/ml *cis*-, but not *trans*-UCA resulted in a decreased proliferative response to anti-CD3 stimulation. This is in contrast to the finding of Holan *et al*, that cells incubated in *cis*-UCA for 12 h or less prior to stimulation showed no significant suppression of proliferation. The shorter time period required for a significant response in the present study could relate to the different mouse strain used. Streilein and Bergstresser (1988) demonstrated that some mouse strains (such as C57BL/6 and C3H/HeN) are more susceptible to UV-induced suppression of CH than others (BALB/c, C3H/HeJ), which require exposure to high doses of UV for immunosuppression to occur. Kurimoto and Streilein (1992) demonstrated that for *cis*-UCA induced suppression of CH, injection of low doses of *cis*-UCA was sufficient to cause suppression in UVB-susceptible mice, while UVB-resistant mice required higher doses of *cis*-UCA for CH suppression. Thus, spleen cells from the 'UVB-susceptible' strain C3H/HeN used in the present study may be more susceptible to *cis*-UCA than spleen cells from the 'UVB-resistant' BALB/c mice.

Alternatively, the shorter pre-incubation time required for suppression of the proliferative response in the present study may be due to the different source of *cis*-UCA used. The *cis*-UCA used by Holan *et al* was a mixture of 50-56 % *cis*-UCA and 44-50 % *trans*-UCA; it was produced by *in vitro* irradiation of *trans*-UCA. In the current study, the *cis*-UCA was a 99% pure preparation; therefore, the use of 200 µg/ml *cis*-UCA gave a *cis*-UCA concentration of approximately double that of Holan *et al*,

which may have reduced the pre-incubation time required for suppression of the proliferative response.

Incubation of spleen cells in *cis*-UCA for 24 h prior to stimulation resulted in a more suppressed response compared with cells incubated for only 2 h. The suppressive effect of 24 h pre-incubation in *cis*-UCA was generally seen upon repetition of the experiment; however, it was often accompanied by a decrease in the background proliferation of unstimulated cells, suggesting that the *cis*-UCA concentration used was toxic to the cells. Pre-incubation in only 100 µg/ ml *cis*-UCA, which gave a similar actual *cis*-UCA concentration to that used by Holan *et al*, had no effect on anti-CD3-stimulated proliferation of spleen cells. Interestingly, pre-incubation of spleen cells in a mixture of *cis*- and *trans*-UCA, which would have given similar concentrations of the isomers as were present in the '*cis*-UCA' cultures used by Holan *et al*, suppressed the proliferative response without affecting the unstimulated cells. Thus it may be that for suppression of spleen cell proliferation, both the presence of *cis*- and *trans*-UCA is required. Using a combination of both isomers is perhaps more relevant to the *in vivo* situation; following UVB exposure of mice, the percentage of *cis*-UCA in the skin increases from 4.7 to approximately 30, and then gradually declines (Norval *et al*, 1988).

Proliferation of T cells in response to soluble CD3 antibody requires the presence of APC (Tsoukas *et al*, 1985), therefore the decreased proliferation could have been due to an effect of UCA on the co-stimulatory capacity of APC. However, Holan *et al* (1998) demonstrated that the decrease in proliferation of spleen cells incubated in *cis*-UCA was due to IL-10 release by CD4⁺ T cells. Spleen cells incubated in *cis*-UCA also showed increased expression of IL-10 mRNA compared with controls. In contrast, a

study by Bi *et al* (1999) demonstrated that incubation of human lymphocytes in *cis*-UCA during PHA stimulation suppressed IL-10 mRNA expression compared with controls.

In the present study, an attempt was made to assess the effect of *cis*-UCA incubation on IL-10 mRNA expression. Spleen cells cultured with or without anti-CD3, in the presence or absence of *cis*- or *trans*-UCA all had similar detectable levels of IL-10 mRNA. RNA extracted from spleen cells cultured with anti-CD3 was incubated with IL-10 and actin primers to demonstrate that there was no contamination of the cDNA mixture with genomic DNA. It is possible that the number of amplification cycles used (40) was too many to detect a difference in yield. Use of a range of cycle numbers to determine the optimum cycle number, or use of real-time PCR might have shown a difference in IL-10 production in *cis*-UCA incubated spleen cells in this study.

6.4 - Summary of Chapter 6

Treatment of mice with *cis*-UCA for a period of 3 weeks had no effect on the outgrowth of FSA cells injected into the treated skin. In mice irradiated for 3 weeks with broad-band UVB prior to injection of FSA cells into the irradiated skin, intraperitoneal injection of a monoclonal antibody to *cis*-UCA prior to each irradiation had no effect on the enhanced outgrowth of FSA cells seen in UVB-exposed mice. Therefore, despite being a mediator of some aspects of UVB-induced immunosuppression, *cis*-UCA does not have an important role in UVB-enhanced tumour outgrowth.

Pre-incubation of spleen cells in *cis*-UCA suppressed subsequent anti-CD3 induced proliferation, compared with cells incubated alone, or in *trans*-UCA; this

suppression was dependent on the concentration of *cis*-UCA, and was not consistent. Incubation of spleen cells in a mixture of *cis* and *trans*-UCA reduced the concentration of *cis*-UCA needed for suppression, suggesting *trans*-UCA is required for *cis*-UCA induced immunosuppression following UV exposure.

Chapter 7

Final Discussion and Summary

UVR is mutagenic, and damages DNA leading to transformation of cells, which can result in skin cancer. In 1974, Kripke demonstrated that UV-induced skin tumours were antigenic, such that, when transplanted from the primary host to syngeneic recipients, these tumours failed to grow, and regressed. This raised questions as to how the tumours were able to develop in the primary host, and it was subsequently found that UV exposure of the recipients prior to tumour transplantation prevented rejection of the tumour, and allowed tumour outgrowth; this susceptibility to tumour outgrowth could be transferred from irradiated mice to naïve mice using spleen and lymph node cells (Fisher and Kripke, 1977).

Following this discovery, a large number of studies have investigated the pathways involved in UV-induced immunosuppression, and a variety of effects of UV on immune parameters have been demonstrated, including alterations in number and function of APC, changes in cytokine profiles, and development of T regulatory cells capable of transferring suppression to other animals. However the mechanisms involved in suppression of immune responses to tumours by UV are still not entirely understood, and in the present study, UV-enhanced tumour outgrowth was assessed in relation to UV-induced immunosuppression. A FSA model was used, where FSA cells were injected into the skin, resulting in tumour formation as early as 3 days post-injection. This was a convenient method of assessing the effects of UV on tumour growth. However, tumour-bearing mice were killed when the tumours showed signs of ulceration, usually at 3-4 weeks post-injection. In a photocarcinogenesis model, mice

irradiated 3 times per week for 5 months did not show tumour development until 18 weeks from the start of the irradiation protocol (Beissert *et al*, 2001). Thus the 3-4 weeks of tumour growth in the present study was a very limited time span in terms of tumour formation. During photocarcinogenesis, neoplastic cells undergo a series of genetic mutations, which can promote their survival and growth; these aspects of tumour growth would not have occurred in the present study, which used FSA cells from a previously established tumour.

Using 3 different lamps, broad-band UVB, narrowband UVB (TL01) and UVA, it was demonstrated that irradiation of mice twice a week for 3 weeks with suberythemal doses of UVB or UVA suppressed the CH response to oxazalone, and decreased the number of epidermal LC. As has been suggested in previous studies, these results indicate that, in the skin of irradiated mice, the reduction in the number of available APC to take up hapten may lead to a decrease in the ability to induce a CH response.

It is thought that the loss of epidermal LC following UVB exposure is due to migration of LC to the DLN. In this and previous studies (Moodycliffe *et al*, 1992; Duthie *et al*, 2000) an increase in DC numbers in the DLN was observed after a single UVB exposure, with a maximum increase at 42 h. However in the present study, it was demonstrated that after a 3 week period of irradiation, the number of DC in the DLN was decreased compared with control mice. Increased levels of apoptosis in LN cells from irradiated mice were not demonstrated. It may be that UV-damaged APC that migrate to the DLN apoptose, resulting in decreased levels of available professional APC in these mice. It has been demonstrated that after a single UVB exposure, very few LC are apoptotic (Okamoto *et al*, 1999), however, the multiple UV exposures used in

the present study may increase the level of apoptosis to significant levels. Following a single UVB exposure, the epidermis is repopulated with B7 deficient LC from the hair follicle (Gilliam *et al*, 1998). These phenotypically altered LC may be more susceptible to UVB-induced apoptosis, after subsequent UV exposures.

Mice irradiated with the broad-band UVB lamp prior to FSA cell injection showed increased outgrowth of subsequently injected FSA cells, however irradiation of mice with the UVA-I (above 340 nm) or TL01 (311 nm) lamps prior to FSA cell injection did not show increased tumour outgrowth. It may be that the wavelengths that cause enhanced tumour outgrowth are the shorter UVB wavelengths (280-305 nm).

Although only the broad-band UVB lamp caused increased outgrowth of injected FSA cells, both UVB and UVA lamps caused LC loss and suppression of CH. Thus the reduction in LC numbers could not be used to predict the induction of susceptibility to tumour cell injection. It is possible that UVB exposure had other effects on the LC, such as alterations in production of cytokines, or expression of cell surface molecules involved in induction of effector T cell responses. Sluyter and Halliday (2000) demonstrated that mice were susceptible to tumour cell injection not at the time of LC loss from the epidermis, but at the time inflammatory cells appeared in the irradiated skin. Previous studies have demonstrated that CD11b⁺ macrophages that migrate into UV exposed human skin produce IL-10 (Kang *et al*, 1994), and that UV-induced suppression of CH in mice could be prevented by treatment with anti-CD11b antibody (Hammerberg *et al*, 1996). An attempt was made to assess the number of CD11b⁺ macrophages in tumours from UVB-exposed and unirradiated mice, by flow cytometry. However, there was no consistent difference in numbers of CD11b⁺ expressing cells between control and irradiated mice. Irradiating mice using the UV

protocol of the present study, and treatment with anti-CD11b antibody, followed by FSA cell injection, would have indicated whether these inflammatory macrophages contribute to UV-enhanced tumour outgrowth.

Studies of the effect of UV-exposure of mice prior to injection of syngeneic tumour cells have mainly used high doses of UV, with each exposure well in excess of a single MED (approximately 4-10 MED) (de Fabo and Kripke, 1980; Romerdahl *et al*, 1988). The present study demonstrated that exposure of mice to a suberythemal dose (1200 J/m^2) of broad-band UVB twice a week for 3 weeks resulted in increase outgrowth of subsequently injected FSA cells. Thus, even suberythemal doses of UV appear to result in suppression of immune responses to skin tumours in mice. Burning of the skin following sun exposure is the most easily recognised indicator of sun damage in humans; however, it may be that exposure to UV doses that are not sufficient to cause sunburn can contribute to the pathogenesis of skin cancer. Use of a solar-simulating UV source in place of the broad-band UVB source employed in the present study would indicate the potential of sun exposure to suppress immune responses to skin tumours.

The FSA cells have been shown previously to be moderately immunogenic in C3H/HeN mice, and in the present study assessment of tumour-bearing mice demonstrated increased DLN cell number, and increased *in vitro* spontaneous proliferation of the DLN cells, compared with tumour-free mice. It is thought that both CD4^+ and CD8^+ T cells are important in anti-tumour immune responses; assessing the phenotype of the cells from the tumour-bearing mice that were proliferating *in vitro* could have indicated whether a particular T cell subset was responding to tumour cell injection. Flow cytometry of DLN cells freshly isolated from tumour-bearing mice

showed both increased numbers of MHC class II⁺ cells, and increased levels of MHC class II expression compared with DLN cells from tumour-free controls. The MHC class II positive cells are probably dendritic cells or other APC, suggesting that there is increased antigen-presentation by DLN cells from tumour-bearing mice.

The increase in number, spontaneous proliferation and MHC class II expression of DLN cells seen in tumour-bearing mice was generally not as great in mice that had been irradiated over a 3 week period prior to FSA cell injection. This indicated that the immune response to the FSA cells was suppressed in mice that had been exposed to UV, which may have allowed the increased tumour outgrowth observed in these mice. A seasonality of presentation of melanoma, BCC and SCC has been demonstrated, with a peak in the summer and autumn months (Swerdlow, 1985). It has been suggested that this peak is due to increased UV exposure during the summer, which increases tumour outgrowth (Romerdaal *et al*, 1988), possibly by suppressing immune responses to these tumours.

Previous studies have demonstrated the presence of transferable regulatory cells in the lymphoid organs of irradiated mice that can suppress immune responses to skin tumours, leading to increased tumour outgrowth. Two different cell types have been proposed to exert this regulatory effect; IL-4-producing NKT cells (Moodycliffe *et al*, 2000), and CTLA-4⁺ T cells that secrete high levels of IL-10 and IFN γ (Beissert *et al*, 1999). In the present study, the percentage of CD4⁺ cells was similar in the DLN of irradiated and unirradiated tumour-bearing mice. The conflicting results of Moodycliffe *et al* and Beissert *et al* could have been further investigated by isolation of any regulatory cells that allow the increased outgrowth of FSA cells in the current study. Measurement by ELISA of cytokines in supernatant following *in vitro* stimulation, or

two-colour flow cytometry, could be used to detect any differences in cytokine expression by DLN T cells from irradiated and unirradiated tumour-bearing mice. It is important to determine the nature of the regulatory cells involved in UV-induced suppression of anti-tumour immune responses so that the effects of these cells can be prevented, to allow the host to develop a protective immune response.

IL-10 has been implicated as a mediator of UV-induced immunosuppression and in one experiment, IL-10 production by DLN cells from irradiated and unirradiated tumour-bearing mice was measured. It was found that DLN cells from unirradiated tumour-bearing mice produced higher amounts of IL-10 after 48 h culture, while DLN cells from irradiated tumour bearing mice had higher levels of IL-10 in the supernatant after 96 h culture. In the present study, only limited data were acquired, therefore no conclusions could be drawn as to the role of IL-10 in suppression of anti-tumour immune responses. Won Byeon *et al* (1998) demonstrated that treatment of mice topically with *Aloe barbadensis* extracts after irradiation restored DTH responses and suppressed IL-10 production in UV-exposed epidermis; however treatment of mice with *Aloe barbadensis* after each exposure during a 12 week irradiation protocol did not prevent UV-enhanced outgrowth of tumour cells (Strickland *et al*, 2001). IL-12 is thought to prevent UV-induced immunosuppression partially by restriction of IL-10 production by irradiated keratinocytes (Schmitt *et al*, 2000). However in the present study IL-12 treatment failed to protect against UV-enhanced tumour outgrowth. Treatment of mice with anti-IL-10 antibody during a 3 week UV-exposure protocol, followed by tumour cell injection, would indicate whether IL-10 is an important mediator of UV-induced suppression of anti-tumour responses.

An attempt was made to determine the phenotype of host cells that infiltrated the FSA tumours, but this was unsuccessful. Use of a mechanical method of tumour disaggregation in conjunction with enzymatic digestion might aid the recovery of host cells from the tumours, for flow cytometric analysis. It has been observed that the majority of infiltrating cells in human skin tumours are T cells (Habets *et al*, 1988), with significant numbers of macrophages also present. However the cell types that are most important for tumour cell destruction are still not completely defined. Identifying any differences in infiltrating cells present in tumours in mice irradiated prior to tumour cell injection, which are less able to control tumour growth, compared with unirradiated mice, could indicate which cells play an important role in tumour destruction. Additionally, comparison of cytokines present in tumour sections from unirradiated and irradiated mice, by immunohistochemistry or *in situ* hybridisation, could reveal the cytokines that would be most effective in promoting anti-tumour responses.

Reeve *et al* (1989) demonstrated that topical application of UCA during photocarcinogenesis in mice increased tumour yield, and Beissert *et al* (2001) showed that injection of a monoclonal antibody specific for *cis*-UCA during photocarcinogenesis reduced the probability of tumour development. *Cis*-UCA has a number of immunosuppressive properties; therefore it is likely that the effects of *cis*-UCA in those previous studies were related to a role in suppression of immune responses to developing skin tumours. Thus, in the present study, it was expected that *cis*-UCA would have a role in UV-enhanced outgrowth of tumour cells injected into mice irradiated over a 3 week period. However, topical application of *cis*-UCA for 3 weeks had no effect on tumour outgrowth in mice, while injection of *cis*-UCA antibody did not reverse UV-enhanced outgrowth of the FSA cells. It is possible that *cis*-UCA

causes increased tumour development during photocarcinogenesis via a mechanism other than immunosuppression. *Cis*-UCA may affect the level of DNA damage sustained during photocarcinogenesis; it is possible that some metabolic products of *cis*-UCA could increase DNA damage in irradiated mice. Alternatively, *cis*-UCA may act to suppress immune responses to tumour cells in the early stages of development, but is not involved in UV-induced suppression of immune responses to tumour cells that are injected following 3 weeks UV exposure. The results of this study suggest that *cis*-UCA is involved in the initiation stage of tumour development, and is not important in tumour progression.

The results of the present study suggest that another photoreceptor may be important for induction of suppression of immune responses to injected tumour cells. DNA damage by UV has been reported as another mechanism by which UV-induced immunosuppression is initiated. Treatment of xeroderma pigmentosum patients for one year topically with T4 endonuclease V, to repair DNA damage, decreased the rate of development of actinic keratoses and BCC (Yarosh *et al*, 2001). However, this effect could have been due to a decreased induction of neoplastic cells, rather than preventing the immunosuppressive effects of DNA damage. Immune responses in irradiated mice have been restored by repairing DNA damage using endonucleases (Wolf *et al*, 1995). This method could be employed to determine whether DNA damage has a role in the UV-enhanced tumour outgrowth seen in the present study.

Studies in mice and humans imply that mast cells may be involved in suppression of immune responses to BCC (Matheson and Reeve, 1991; Grimbaldston *et al*, 2000). *Cis*-UCA is thought to mediate mast cell degranulation via sensory nerve innervation. Keratinocyte-derived NGF is upregulated following UVB exposure, and

may also cause mast cell degranulation via neuropeptide release by sensory nerves; this mechanism appears to be independent of the action of *cis*-UCA (Townley *et al*, 2002). Anti-NGF antibodies could be used to assess whether this mechanism has a role in UV-enhanced tumour outgrowth.

Another possible mediator of UV-induced immunosuppression is platelet-activating factor (PAF) which is produced by irradiated keratinocytes, and which upregulates immunomodulatory cytokines, including PGE₂. Treatment of mice with PAF suppressed DTH to *Candida albicans*, and injection of a PAF receptor antagonist blocked UV-induced suppression of DTH (Walterscheid *et al*, 2002). Walterscheid *et al* proposed that PAF synthesis is induced during cellular repair of UV-induced DNA damage, causing immunosuppression. PAF-receptor antagonists can also reverse *cis*-UCA induced suppression, and Walterscheid *et al* suggested that *cis*-UCA works in concert with UV-induced DNA damage to amplify PAF synthesis. The results from the present study indicate that *cis*-UCA is not the main photoreceptor for UV-induced suppression of anti-tumour responses, suggesting a role for DNA-damage, possibly via PAF induction. *Cis*-UCA may act to potentiate this effect in the long-term photocarcinogenesis studies by Beissert *et al* (2001), and Reeve *et al* (1989), however during the short-term UV exposure model used in the present study, the potentiating effect of *cis*-UCA was too slight to measure. Figure 7.1 illustrates some of the pathways involved in UV-induced immune suppression, demonstrating the possible role for *cis*-UCA in potentiating the mechanisms by which immune suppression is initiated.

The immunogenicity of many UV-induced skin cancers offers the potential for treatment of these cancers by manipulation of the immune system of the host. Knowledge of which cells are important for tumour destruction could guide the

development of mechanisms to induce an effective host response; comparison of immune responses in irradiated and unirradiated tumour-bearing mice could indicate which cells are important in the anti-tumour immune response. The demonstration of UV-induced regulatory T cells offers a possible target for therapy of skin cancer patients; understanding the nature and mechanism of action of these cells could lead to prevention of their action, such that tumour-destructive immune responses are not dampened by these regulatory cells.

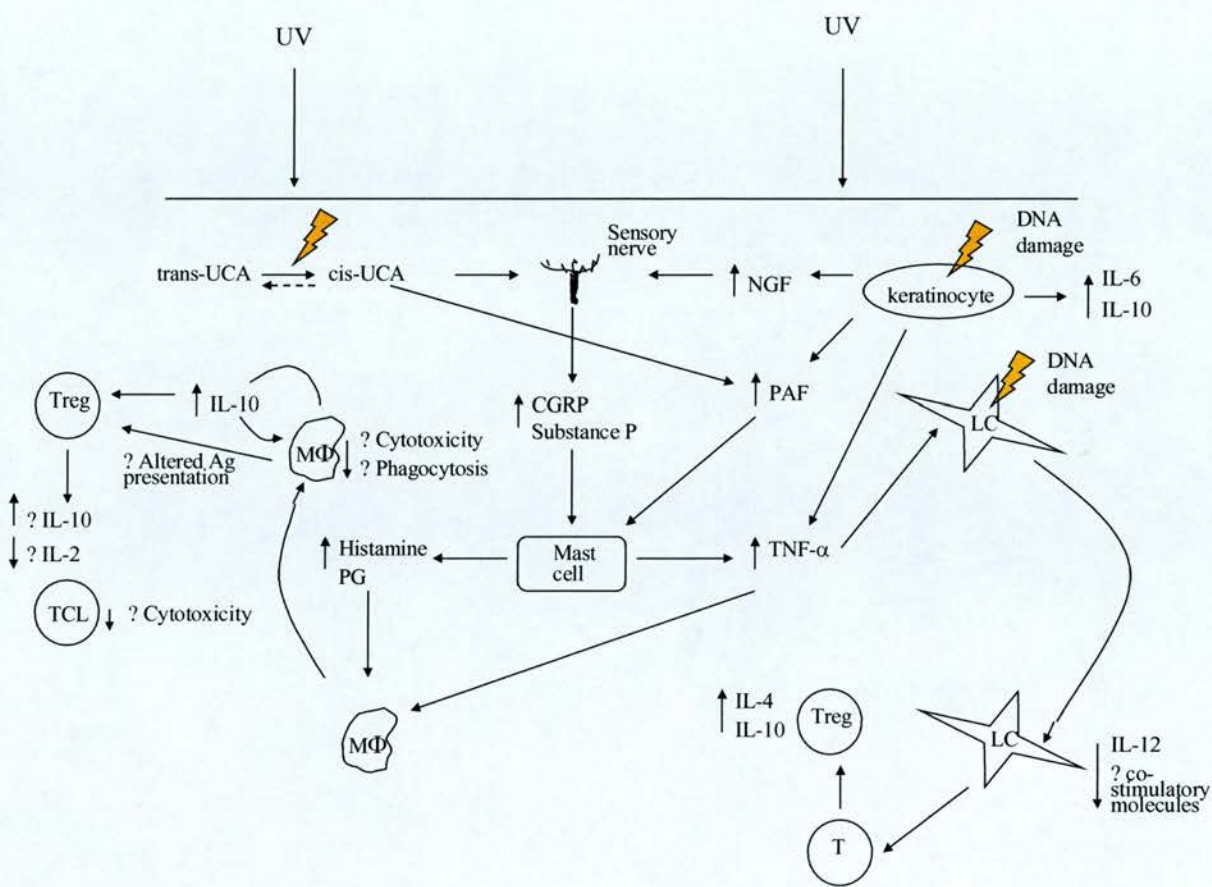


Figure 7.1. Mechanisms involved in UV-induced immunosuppression. TCL = cytotoxic T lymphocyte.

Treg = regulatory T cell. MΦ = macrophage. PG = prostaglandin. Ag = antigen.

Bibliography

Aberer W, Schuler G, Stingl G, Honigsmann H and Wolff K (1981) Ultraviolet light depletes surface markers of Langerhans cells. *J. Invest. Dermatol.* **76**, 202-210.

Alcalay J and Kripke ML (1990) Antigen presenting activity of draining lymph node cells from mice painted with a contact allergen during ultraviolet carcinogenesis. *J. Immunol* **6**, 1717-1721.

Anglin JH, Bever AT, Everet MA and Lamb JH (1961) Ultraviolet-light induced alterations in urocanic acid *in vivo*. *Biochim. Biophys. Acta* **53**, 408-409.

Aubin F, Donawho CK and Kripke ML (1991) Effect of psoralen plus ultraviolet A radiation on *in vivo* growth of melanoma cells. *Cancer Res.* **51**, 5893-5897.

Bacci S, Alard P and Streilein JW (2001) Evidence that ultraviolet B radiation transiently inhibits emigration of Langerhans cells from exposed epidermis, thwarting contact hypersensitivity induction. *Eur. J. Immunol.* **31**, 3588-3594.

Beissert S, Hosoi J, Grabbe S, Asahina A and Granstein RD (1995) IL-10 inhibits tumor antigen presentation by epidermal antigen-presenting cells. *J. Immunol.* **154**, 1280-1286.

Beissert S, Hosoi JC, Kuhn R, Rajewsky K, Muller W and Granstein RD (1996) Impaired immunosuppressive response to ultraviolet radiation in interleukin-10 deficient mice. *J. Invest. Dermatol.* **107**, 552-557.

Beissert S, Mohammad T, Torri H, Lonati A, Yan ZM, Morrison H and Granstein RD (1997) Regulation of tumor antigen presentation by urocanic acid. *J. Immunol.* **159**, 92-96.

Beissert S, Bluestone JA, Mindt I, Voskort M, Metze D, Mehling A, Luger TA, Schwarz T and Grabbe S (1999) Reduced ultraviolet-induced carcinogenesis in mice with a functional disruption in B7-mediated costimulation. *J. Immunol.* **163**, 6725-6731.

Beissert S, Ruhlemann D, Mohammad T, Grabbe S, El-Ghorr A, Norval M, Morrison H, Granstein RD and Schwarz T (2001) IL-12 prevents the inhibitory effects of *cis*-urocanic acid on tumor antigen presentation by Langerhans cells: Implications for photocarcinogenesis. *J. Immunol.* **167**, 6232-6238.

Bennicelli JL and Guerry D 4th (1993) Production of multiple cytokines by cultured human melanomas. *Exp. Dermatol.* **2**, 186-190.

Berg EL, Yoshino T, Rott LS, Robinson MK, Warnock RA, Kishimoto TK, Picker LJ and Butcher EC (1991) The cutaneous lymphocyte antigen is a skin lymphocyte homing receptor for the vascular lectin endothelial cell-leukocyte adhesion molecule 1. *J. Exp. Med.* **174**, 1461-1466.

Berg RJ, van Kranen HJ, Rebel HG, de Vries A, van Vloten WA, Van Kreijl CF, van der Leun JC and de Gruijl FR (1996) Early p53 alterations in mouse skin carcinogenesis by UVB radiation: immunohistochemical detection of mutant p53 protein in clusters of preneoplastic epidermal cells. *Proc. Natl. Acad. Sci. USA* **93**, 274-278.

Bergfelt L, Larko O and Lindberg M (1992) Density and morphology of Langerhans cells in basal cell carcinomas of the face and trunk. *Br. J. Dermatol.* **127**, 575-579.

Bergfelt L, Emilson A, Lindberg M and Scheynius A (1994) Quantitative and 3-dimensional analysis of Langerhans cells in basal cell carcinoma. A comparative study using light microscopy and confocal laser scanning microscopy. *Br. J. Dermatol.* **130**, 273-280.

- Bestak R and Halliday GM (1996) Chronic low-dose UVA irradiation induces local suppression of contact hypersensitivity, Langerhans cell depletion and suppressor cell activation in C3H/HeJ mice *Photochem. Photobiol.* 64, 969-974.
- Bi ZG, Xia JP and Ni L (1999) Effects of ultraviolet-irradiated urocanic acid on IL-10 secreted by T lymphocytes. *Chin. Med. J. (Engl.)* 112, 509-511.
- Birbeck MS, Breathnach AS and Everall JD (1961) An electron microscopic study of basal melanocytes and high level clear cells (Langerhans cells) in vitiligo. *J. Invest. Dermatol.* 37, 51-63.
- Booth JC, Hannington G, Aziz TA and Stern H (1979) Comparison of enzyme-linked immunosorbent assay (ELISA) technique and complement-fixation test for estimation of cytomegalovirus IgG antibody. *J. Clin. Pathol.* 32, 122-127.
- Borkowski J (2000) Homogenisation of the Belsk UV-B Series (1976-1997) and Trend Analysis. *J. Geophys. Res.* 105, 4873-4878.
- Bos JD and Kapsenberg ML (1986) The skin immune system (SIS): its cellular constituents and their interactions. *Immunol. Today* 7, 235-240.
- Boyle J, MacKie RM, Briggs JD, Junor BJ and Aitchison TC (1984) Cancer, warts, and sunshine in renal transplant patients. A case-control study. *Lancet* 1, 702-705.
- Brash DE, Rudolph JA, Simon JA, Lin A, McKenna GJ, Baden HP, Halperin AJ and Ponten J (1991) A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc. Natl. Acad. Sci. USA* 88, 10124-10128.
- Brunda MJ, Luistro L, Warriar RR, Wright RB, Hubbard BR, Murphy M, Wolf SF and Gately MK (1993) Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J. Exp. Med.* 178, 1223-1230.

Bucana CD, Tang JM, Dunner K Jr, Strickland FM and Kripke ML (1994) Phenotypic and ultrastructural properties of antigen-presenting cells involved in contact sensitization of normal and UV-irradiated mice. *J. Invest. Dermatol.* **102**, 928-933.

Caforio AL, Belloni Fortina A, Gambino A, Piaserico S, Feltrin G, Tona F, Pompei E, Testolin L, Gai F, Angelini A, Casarotto D and Peserico A (2001) High rejection score in the first year and risk of skin cancer in heart transplantation. *Transplant. Proc.* **33**, 1608-1609.

Campbell C, Quinn AG and Rees JL (1993) Codon 12 Harvey-*ras* mutations are rare events in non-melanoma human skin cancer. *Br. J. Dermatol.* **128**, 111-114.

Campton K, Ding WH, Yan ZM, Ozawa H, Seiffert K, Miranda E, Lonati A, Beissert S and Granstein RD (2000) Tumor antigen presentation by dermal antigen-presenting cells. *J. Invest. Dermatol.* **115**, 57-61.

Cavanagh LL, Barnetson RS, Basten A and Halliday GM (1997) Dendritic epidermal T-cell involvement in induction of CD8⁺ T cell-mediated immunity against an ultraviolet radiation-induced skin tumor. *Int. J. Cancer* **70**, 98-105.

Chikamatsu K, Eura M, Matsuoka H, Murakami H, Fukiage T and Ishikawa T (1994) The role of major histocompatibility complex expression on head and neck cancer cells in the induction of autologous cytotoxic T lymphocytes. *Cancer Immunol. Immunother.* **38**, 358-364.

Chin L, Tam A, Pomerantz J, Wong M, Holash J, Bardeesy N, Shen Q, O'Hagan R, Pantginis J, Zhou H, Horner JW 2nd, Cordon-Cardo C, Yancopoulos GD and DePinho RA (1999) Essential role for oncogenic Ras in tumour maintenance. *Nature* **400**, 468-472.

Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML and Wyllie AH (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* **362**, 849-852.

Cohen PJ, Cohen PA, Rosenberg SA, Katz SI and Mule JJ (1994) Murine epidermal Langerhans cells and splenic dendritic cells present tumor-associated antigens to primed T cells. *Eur. J. Immunol.* **24**, 315-319.

Cooper KD, Duraiswamy N, Hammerberg C, Allen E, Kimbroughreen C, Dillon W and Thomas D (1993) Neutrophils, differentiated macrophages, and monocyte/macrophage antigen presenting cells infiltrate murine epidermis after UV injury. *J. Invest. Dermatol.* **101**, 155-163.

Cress RD and Holly EA (1997) Incidence of cutaneous melanoma among non-Hispanic whites, Hispanics, Asians, and blacks: an analysis of California cancer registry data, 1988-93. *Cancer Causes Control* **8**, 246-252.

Cruz PD Jr, Leverkus M, Dougherty I, Gleason MJ, Eller M, Yaar M and Gilchrest BA (2000) Thymidine dinucleotides inhibit contact hypersensitivity and activate the gene for tumor necrosis factor alpha-1. *J. Invest. Dermatol.* **114**, 253-258.

Dahl E, Aberg M, Rausing A and Rausing EL (1992) Basal cell carcinoma. An epidemiologic study in a defined population. *Cancer* **70**, 104-108.

Dandie GW, Clydesdale GJ, Radcliff FJ and Müller HK (2001) Migration of Langerhans cells and $\gamma\delta^+$ dendritic cells from UV-B irradiated sheep skin. *Immun. Cell. Biol.* **79**, 41-48.

Danno K, Fujii K, Tachibana T, Toda K and Horio T (1988) Suppressed histamine release from rat peritoneal mast cells by ultraviolet B irradiation: decreased diacylglycerol formation as a possible mechanism. *J. Invest. Dermatol.* **90**, 806-809.

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W *et al* (2002) Mutations of the BRAF gene in human cancer. *Nature* **417**, 949-954.

Daynes RA, Spellman CW, Woodward JG and Stewart DA (1977) Studies into the transplantation biology of ultraviolet light-induced tumors. *Transplantation* **23**, 343-348.

De Fabo EC and Kripke ML (1980) Wavelength dependence and dose rate independence of UV radiation-induced immunologic unresponsiveness of mice to a UV-induced fibrosarcoma. *Photochem. Photobiol.* **32**, 183-188.

De Fabo EC and Noonan FP (1983) Mechanism of immune suppression by ultraviolet irradiation *in vivo*. I. Evidence for the existence of a unique photoreceptor in skin and its role in photoimmunology. *J. Exp. Med.* **158**, 84-98.

de Fine Olivarius F, Lock-Andersen J, Larsen FG, Wulf HC, Crosby J and Norval M (1998) Urocanic acid isomers in patients with basal cell carcinoma and cutaneous malignant melanoma. *Br. J. Dermatol.* **138**, 986-992.

de Gruijl FR, Sterenborg HJCM, Forbes PD, Davies RE, Cole C, Kelfkens G, van Weelden H, Slaper H and van der Leun JC (1993) Wavelength dependence of skin cancer induction by ultraviolet irradiation of albino hairless mice. *Cancer Res.* **53**, 53-60.

de Simone C, Masini C, Cattaruzza MS, Guerriero C, Cerimele D and Norval M (2001) Urocanic acid isomers in patients with non-melanoma skin cancer. *Br. J. Dermatol.* **144**, 858-861.

Denfeld RW, Tesmann JP, Dittmar H, Weiss JM, Schopf E, Weltzien HU and Simon JC (1998) Further characterization of UVB radiation effects on Langerhans cells: altered expression of the costimulatory molecules B7-1 and B7-2. *Photochem. Photobiol.* **67**, 554-560.

De Vita F, Orditura M, Galizia G, Romano C, Lieto E, Iodice P, Tuccillo C and Catalano G (2000) Serum interleukin-10 is an independent prognostic factor in advanced solid tumors. *Oncol. Rep.* **7**, 357-361.

de Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C, Johnson K, Kastelein R, Yssel H and de Vries JE (1991) Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J. Exp. Med.* **174**, 915-924.

Donawho CK and Kripke ML (1991) Enhanced growth of murine melanoma in ultraviolet-irradiated skin is associated with local inhibition of immune effector mechanisms. *Cancer Res.* **51**, 4176-4181.

Donawho CK and Kripke ML (1992) Lack of correlation between UV-induced enhancement of melanoma development and local suppression of contact hypersensitivity. *Exp. Dermatol.* **1**, 20-26.

Donawho CK, Wolf P and Kripke ML (1994) Enhanced development of murine melanoma in UV irradiated skin – UV dose response, waveband dependence and relation to inflammation. *Melanoma Res.* **4**, 93-100.

Donawho CK, Muller HK, Bucana CD and Kripke ML (1996) Enhanced growth of murine melanoma in ultraviolet irradiated skin is associated with local inhibition of immune effector mechanisms. *J. Immunol.* **157**, 781-786.

Dustin ML, Singer KH, Tuck DT and Springer TA (1988) Adhesion of T lymphoblasts to epidermal keratinocytes is regulated by interferon gamma and is mediated by intercellular adhesion molecule 1 (ICAM-1). *J. Exp. Med.* **167**, 1323-1340.

Duthie MS, Kimber I, Dearman RJ and Norval M (2000) Differential effects of UVA1 and UVB radiation on Langerhans cell migration in mice. *J. Photochem. Photobiol: B-Biol.* **57**, 123-131.

Elsasser-Beile U, von Kleist S, Stahle W, Schurhammer-Fuhrmann C, Monting JS and Gallati H (1993) Cytokine levels in whole blood cell cultures as parameters of the cellular immunologic activity in patients with malignant melanoma and basal cell carcinoma. *Cancer* **71**, 231-236.

Elder DE (1995) Skin cancer. Melanoma and other specific nonmelanoma skin cancers. *Cancer* **75**, 245-256.

El-Ghorr AA and Norval M (1995) A monoclonal antibody to *cis*-urocanic acid prevents the ultraviolet-induced changes in Langerhans cells and delayed hypersensitivity responses in mice, although not preventing dendritic cell accumulation in lymph nodes draining the site of irradiation and contact hypersensitivity responses. *J. Invest. Dermatol.* **105**, 264-268.

El-Ghorr AA, Norval M, Lappin MB and Crosby JC (1995) The effect of chronic low-dose UVB radiation on Langerhans cells, sunburn cells, urocanic acid isomers, contact hypersensitivity and serum immunoglobulins in mice. *Photochem. Photobiol.* **62**, 326-332.

El-Ghorr AA and Norval M (1997a) The role of interleukin-4 in ultraviolet B light-induced immunosuppression. *Immunology* **92**, 26-32.

El-Ghorr AA and Norval M (1997b) The effect of chronic treatment of mice with urocanic acid isomers. *Photochem. Photobiol.* **65**, 866-872.

- El-Ghorr AA and Norval M (1999) The UV waveband dependencies in mice differ for the suppression of contact hypersensitivity, delayed-type hypersensitivity and *cis*-urocanic acid formation. *J. Invest. Dermatol.* **112**, 757-762.
- Ellis RJ, Wernick G, Zabriskie JB and Goldman LI (1975) Immunologic competence of regional lymph nodes in patients with breast cancer. *Cancer* **35**, 655-659.
- English DR, Armstrong BK, Krickler A, Winter MG, Heenan PJ and Randell PL (1998) Case-control study of sun exposure and squamous cell carcinoma of the skin. *Int. J. Cancer* **77**, 347-353.
- Enk AH and Katz SI (1992) Identification and induction of keratinocyte-derived IL-10. *J. Immunol.* **149**, 92-95.
- Espana A, Redondo P, Fernandez AL, Zabala M, Herreros J, Llorens R and Quintanilla E (1995) Skin cancer in heart transplant recipients. *J. Am. Acad. Dermatol.* **32**, 458-465.
- Fisher DE (1994) Apoptosis in cancer therapy – crossing the threshold. *Cell* **78**, 539-542.
- Fisher MS and Kripke ML (1977) Systemic alteration induced in mice by ultraviolet light irradiation and its relationship to ultraviolet carcinogenesis. *Proc. Natl. Acad. Sci. USA* **74**, 1688-1692.
- Fisher MS, Kripke ML (1982) Suppressor T lymphocytes control the development of primary skin cancers in ultraviolet-irradiated mice. *Science* **216**, 1133-1134.
- Garcia-Hernandez ML, Hernandez-Pando R, Gariglio P and Berumen J (2002) Interleukin-10 promotes B16-melanoma growth by inhibition of macrophage functions and induction of tumour and vascular cell proliferation. *Immunology* **105**, 231-243.

Garssen J, Buckley TL and Van Loveren H (1998) A role for neuropeptides in UVB-induced systemic immunosuppression. *Photochem. Photobiol.* **68**, 205-210.

Garssen J, Norval M, Crosby J, Dortant P and Van Loveren H (1999) The role of urocanic acid in UVB-induced suppression of immunity to *Trichinella spiralis* infection in the rat. *Immunology* **96**, 298-306.

Garssen J, Vandebriel RJ, de Gruijl FR, Wolvers DAW, Van Dijk M and Fluitman A (1999) UVB exposure-induced systemic modulation of Th1- and Th2-mediated immune responses. *Immunology* **97**, 506-514.

Gibbs NK, Norval M, Traynor NJ, Wolf M, Johnson BE and Crosby J (1993) Action spectra for the trans to cis photoisomerisation of urocanic acid in vitro and in mouse skin. *Photochem. Photobiol.* **57**, 584-590.

Gibbs NK, McLoone PR and Simics E (1997) An action spectrum for urocanic acid isomerisation in human skin *in vivo*. *Photochem. Photobiol.* **65**, 103S.

Gilliam AC, Kremer IB, Yuichi Y, Stevens SR, Tootell E, Teunissen MB, Hammerberg C and Cooper KD (1998) The human hair follicle: A reservoir of CD40⁺ B7-deficient Langerhans cell that repopulate epidermis after UVB exposure. *J. Invest. Dermatol.* **110**, 422-427.

Gilmour JW, Vestey JP, George S and Norval M (1993) Effect of phototherapy and urocanic acid isomers on natural killer cell function. *J. Invest. Dermatol.* **101**, 169-174.

Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, Hobby P, Sutton B, Tigelaar RE and Hayday AC (2001) Regulation of cutaneous malignancy by gammadelta T cells. *Science* **294**, 605-609.

Glass MJ, Bergstresser PR, Tigelaar RE and Streilein JW (1990) UVB radiation and DNFB skin painting induce suppressor cells universally in mice. *J. Invest. Dermatol.* **94**, 273-278.

Godar DE, Wengraitis SP, Shreffler J and Sliney DH (2001) UV doses of Americans. *Photochem. Photobiol.* **73**, 621-629.

Goettsch W, Hurks HM, Garssen J, Mommaas AM, Slob W, Hoekman J, Pierik F, Roholl PJ and Van Loveren H (1998) Comparative immunotoxicology of ultraviolet B exposure I. Effects of *in vitro* and *in situ* ultraviolet B exposure on the functional activity and morphology of Langerhans cells in the skin of different species. *Br. J. Dermatol.* **139**, 230-238.

Grabbe S, Bruvers S, Gallo RL, Knisely TL, Nazareno R and Granstein RD (1991) Tumour antigen presentation by murine epidermal cells. *J. Immunol.* **146**, 3656-3661.

Grabbe S, Bruvers S, Lindgren AM, Hosoi J, Tan KC and Granstein RD (1992) Tumor antigen presentation by epidermal antigen-presenting cells in the mouse: modulation by granulocyte-macrophage colony-stimulating factor, tumor necrosis factor alpha, and ultraviolet radiation. *J. Leukoc. Biol.* **52**, 209-217.

Grabbe S, Bruvers S, Beissert S and Granstein RD (1994) Interferon-gamma inhibits tumor antigen presentation by epidermal antigen-presenting cells. *J. Leukoc. Biol.* **55**, 695-701.

Green A, Williams G, Neale R, Hart V, Leslie D, Parsons P, Marks GC, Gaffney P, Battistutta D, Frost C, Lang C and Russell A (1999) Daily sunscreen application and betacarotene supplementation in prevention of basal-cell and squamous-cell carcinomas of the skin: a randomised controlled trial. *Lancet* **354**, 723-729.

Greene MH, Young TI and Clark WH Jr (1981) Malignant melanoma in renal-transplant recipients. *Lancet* **1**, 1196-1199.

- Grimbaldeston MA, Skov L, Baadsgaard O, Skov BG, Marshman G, Finlay-Jones JJ and Hart PH (2000) Communications: high dermal mast cell prevalence is a predisposing factor for basal cell carcinoma in humans. *J. Invest. Dermatol.* **115**, 317-320.
- Grimwood RE, Glanz SM and Siegle RJ (1988) Transplantation of human basal cell carcinoma to C57/BALB/C bgJ/bgJ-nu/nu (beige-nude) mouse. *J. Dermatol. Surg. Oncol.* **14**, 59-62.
- Gruner S, Diezel W, Stoppe H, Oesterwitz H, Henke W (1992a) Inhibition of skin allograft rejection and acute graft-versus-host disease by *cis*-urocanic acid. *J. Invest. Dermatol.* **98**, 459-462
- Gruner S, Oesterwitz H, Stoppe H, Henke W, Eckert R, and Sonnichsen N (1992b) *Cis*-urocanic acid as a mediator of ultraviolet-light-induced immunosuppression. *Semin. Hematol.* **29**, 102-107.
- Gurish MF, Roberts LK, Krueger GG and Daynes RA (1981) The effect of various sunscreen agents on skin damage and the induction of tumor susceptibility in mice subjected to ultraviolet irradiation. *J. Invest. Dermatol.* **76**, 246-251.
- Habets JM, Tank B, Vuzevski VD, van Reede EC, Stolz E and van Joost T (1988) Characterization of the mononuclear infiltrate in basal cell carcinoma: a predominantly T cell-mediated immune response with minor participation of Leu-7+ (natural killer) cells and Leu-14+ (B) cells. *J. Invest. Dermatol.* **90**, 289-292.
- Haeffner AC, Zepter K, Elmetts CA and Wood GS (1997) Analysis of tumor-infiltrating lymphocytes in cutaneous squamous cell carcinoma. *Arch. Dermatol.* **133**, 585-590.

Hakansson L, Adell G, Boeryd B, Sjogren F and Sjodahl R (1997) Infiltration of mononuclear inflammatory cells into primary colorectal carcinomas: an immunohistological analysis. *Br. J. Cancer* **75**, 374-380.

Halliday GM, MacCarrick GR and Muller HK (1987) Tumour promoters but not initiators deplete Langerhans cells from murine epidermis. *Br. J. Cancer*. **56**, 328-330.

Halliday GM, Reeve VE and Barnetson RS (1991) Langerhans cell migration into ultraviolet light-induced squamous skin tumours is unrelated to antitumour immunity. *J. Invest. Dermatol.* **97**, 830-834.

Hammerberg C, Duraiswamy N and Cooper KD (1996) Reversal of immunosuppression inducible through ultraviolet-exposed skin by *in vivo* anti-CD11b treatment. *J. Immunol.* **157**, 5254-5261.

Harada M, Okamoto T, Omoto K, Tamada K, Takenoyama M, Hirashima C, Ito O, Kimura G and Nomoto K (1996) Specific immunotherapy with tumour-draining lymph node cells cultured with both anti-CD3 and anti-CD28 monoclonal antibodies. *Immunology* **87**, 447-453.

Hardie IR, Strong RW, Hartley LC, Woodruff PW and Clunie GJ (1980) Skin cancer in Caucasian renal allograft recipients living in a subtropical climate. *Surgery* **87**, 177-183.

Hart PH, Jones CA, Jones KL, Watson CJ, Santucci I, Spencer LK and Finlay-Jones JJ (1993) *Cis*-urocanic acid stimulates human peripheral blood monocyte prostaglandin E2 production and suppresses indirectly tumor necrosis factor- α levels. *J. Immunol.* **150**, 4514-4523.

Hart PH, Jaksic A, Swift G, Norval M, El-Ghorr AA and Finlay-Jones JJ (1997) Histamine involvement in UVB- and *cis*-urocanic acid-induced systemic suppression of contact hypersensitivity responses. *Immunology* **91**, 601-608.

Hart PH, Grimbaldston MA, Swift GJ, Jaksic A, Noonan FP and Finlay-Jones JJ (1998) Dermal mast cells determine susceptibility to ultraviolet B-induced systemic suppression of contact hypersensitivity responses in mice. *J. Exp. Med.* **187**, 2045-2053.

Hartevelt MM, Bavinck JN, Kootte AM, Vermeer BJ and Vandenbroucke JP (1990) Incidence of skin cancer after renal transplantation in The Netherlands. *Transplantation* **49**, 506-509.

Herbst RA, Gutzmer R, Matiaske F, Mommert S, Kapp A, Weiss J, Arden KC and Cavenee WK (1997) Further evidence for ultraviolet light induction of CDKN2 (p16(INK4)) mutations in sporadic melanoma *in vivo*. *J. Invest. Dermatol.* **108**, 950.

Hill LL, Perussia B, McCue PA and Korngold R (1994) Effect of human natural killer cells on the metastatic growth of human melanoma xenografts in mice with severe combined immunodeficiency. *Cancer Res.* **54**, 763-770.

Holan V, Kuffova L, Zajicova A, Krulova M, Filipec M, Holler P and Jancarek A (1998) Urocanic acid enhances IL-10 production in activated CD4⁺ T cells. *J. Immunol.* **161**, 3237-3241.

Holman CD, Armstrong BK and Heenan PJ (1983) A theory of the etiology and pathogenesis of human cutaneous malignant melanoma. *J. Natl. Cancer Inst.* **71**, 651-656.

Holme SA, Malinowszky K and Roberts DL (2000) Changing trends in non-melanoma skin cancer in South Wales, 1988-98. *Br. J. Dermatol.* **143**, 1224-1229.

Hoon DS, Bowker RJ and Cochran AJ (1987) Suppressor cell activity in melanoma-draining lymph nodes. *Cancer Res.* **47**, 1529-1533.

Hostetler LW, Romerdahl CA and Kripke ML (1989) Specificity of antigens on UV radiation-induced antigenic tumor cell variants measured *in vitro* and *in vivo*. *Cancer Res.* **49**, 1207-1213.

Howie S and McBride WH (1982) Tumour specific T helper activity can be abrogated by two distinct suppressor cell mechanisms. *Eur. J. Immunol.* **12**, 671-675.

Hua LA, Kagen CN, Carpenter RJ and Goltz RW (1985) HLA and beta 2-microglobulin expression in basal and squamous cell carcinomas of the skin. *Int. J. Dermatol.* **24**, 660-663.

Hug DH and Hunter JK (1991) *Cis*-urocanic acid applied to mouse skin is internalised. Proc. 4th Congress Eur. Soc. For. Photobiology, Amsterdam, The Netherlands, 174.

Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D and Levitsky H (1998) The central role of CD4(+) T cells in the antitumor immune response. *J. Exp. Med.* **188**, 2357-2368.

Hunt MJ, Halliday GM, Weedon D, Cooke BE and Barnetson RS (1994) Regression in basal cell carcinoma: an immunohistochemical analysis. *Br. J. Dermatol.* **130**, 1-8.

Husain Z, Yang QM and Biswas DK (1990) cHa-ras proto-oncogene. Amplification and overexpression in UV-B-induced mouse skin papillomas and carcinomas. *Arch. Dermatol.* **126**, 324-330.

Jackson M, Thomson KE, Laker R, Norval M, Hunter JA and McKenzie RC (1996) Lack of induction of IL-10 expression in human keratinocytes. *J. Invest. Dermatol.* **106**, 1329-1331.

Kaminski MJ, Cruz PD Jr, Bergstresser PR and Takashima A (1993) Killing of skin-derived tumor cells by mouse dendritic epidermal T cells. *Cancer Res.* **53**, 4014-4019.

Kammeyer A, Pavel S, Asghar SS, Bos JD and Teunissen MB (1997) Prolonged increase of *cis*-urocanic acid levels in human skin and urine after single total-body ultraviolet exposures. *Photochem. Photobiol.* **65**, 593-598.

Kammeyer A, Eggelte TA, Overmars H, Bootsma A, Bos JD and Teunissen MB (2001) Oxidative breakdown and conversion of urocanic acid isomers by hydroxyl radical generating systems. *Biochim. Biophys. Acta.* **1526**, 277-285.

Kang KF, Hammerberg C, Meunier L and Cooper KD (1994) CD11b⁺ Macrophages that infiltrate human epidermis after *in vivo* ultraviolet exposure potently produce IL-10 and represent the major secretory source of epidermal IL-10 protein. *J. Immunol.* **153**, 5256-5264.

Kanjilal S, Pierceall WE, Cummings KK, Kripke ML and Ananthaswamy HN (1993) High frequency of p53 mutations in ultraviolet radiation-induced murine skin tumors: evidence for strand bias and tumor heterogeneity. *Cancer Res.* **53**, 2961-2964.

Karagas MR, Greenberg ER, Spencer SK, Stukel TA and Mott LA (1999) Increase in incidence rates of basal cell and squamous cell skin cancer in New Hampshire, USA. New Hampshire Skin Cancer Study Group. *Int. J. Cancer* **81**, 555-559.

Kettering JD, Schmidt NJ and Lennette EH (1977) Improved glycine-extracted complement-fixing antigen for human cytomegalovirus. *J. Clin. Microbiol.* **6**, 647-649.

Khalil Z, Townley SL, Grimbaldston MA, Finlay-Jones JJ and Hart PH (2001) *Cis*-urocanic acid stimulates neuropeptide release from peripheral sensory nerves. *J. Invest. Dermatol.* **117**, 886-891.

Kibitel J, Hejmadi V, Alas L, O'Connor A, Sutherland BM and Yarosh D (1998) UV-DNA damage in mouse and human cells induces the expression of tumor necrosis factor alpha. *Photochem. Photobiol.* **67**, 541-546.

Kielbassa C, Roza L and Epe B (1997) Wavelength dependence of oxidative DNA damage induced by UV and visible light. *Carcinogenesis* **18**, 811-816.

Kiertscher SM, Luo J, Dubinett SM and Roth MD (2000) Tumors promote altered maturation and early apoptosis of monocyte-derived dendritic cells. *J. Immunol.* **164**, 1269-1276.

Kimber I, Mitchell JA and Griffin AC (1986) Development of a murine local lymph node assay for the determination of sensitizing potential. *Food Chem. Toxicol.* **24**, 585-586.

Kimber I, Kinnaird A, Peters SW and Mitchell JA (1990) Correlation between lymphocyte proliferative responses and dendritic cell migration in regional lymph nodes following skin painting with contact-sensitizing agents. *Int. Arch. Allergy Appl. Immunol.* **93**, 47-53.

Kitazawa T and Streilein JW (2000) Studies on delayed systemic effects of ultraviolet B radiation on the induction of contact hypersensitivity, 3. Dendritic cells from secondary lymphoid organs are deficient in interleukin-12 production and capacity to promote activation and differentiation of T helper type 1 cells. *Immunology* **99**, 296-304.

Kivisto K, Punnonen K, Toppari J and Leino L (1996) Urocanic acid suppresses the activation of human neutrophils in vitro. *Inflammation* **20**, 451-459.

Koblish HK, Hunter CA, Wysocka M, Trinchieri G and Lee WMF (1998) Immune suppression by recombinant interleukin (rIL)-12 involves interferon gamma induction of nitric oxide synthase 2 (iNOS) activity: Inhibitors of NO generation reveal the extent of rIL-12 vaccine adjuvant effect. *J. Exp. Med.* **188**, 1603-1610.

Kock A, Schwarz T, Kirnbauer R, Urbanski A, Perry P, Ansel JC and Luger TA (1990) Human keratinocytes are a source for tumor necrosis factor alpha: evidence for synthesis and release upon stimulation with endotoxin or ultraviolet light. *J. Exp. Med.* **172**, 1609-1614.

Kohchiyama A, Oka D and Ueki H (1986) Immunohistologic studies of squamous cell carcinoma: possible participation of Leu-7+ (natural killer) cells as antitumor effector cells. *J. Invest. Dermatol.* **87**, 515-518.

Kölgen W, Both H, van Weelden H, Guikers KLH, Bruijnzeel-Koomen AFM, Knol EF, van Vloten WA and de Gruijl FR (2002) Epidermal Langerhans cell depletion after artificial ultraviolet B irradiation of human skin *in vivo*: Apoptosis versus migration. *J. Invest Dermatol.* **118**, 812-817.

Kondo S, Sauder DN, McKenzie RC, Fujisawa H, Shivji GM, El-Ghorr AA and Norval M (1995) The role of *cis*-urocanic acid in UVB-induced suppression of contact sensitisation. *Immunol. Lett.* **48**, 181-186.

Koranda FC, Loeffler RT, Koranda DM and Penn I (1975) Accelerated induction of skin cancers by ultraviolet radiation in hairless mice treated with immunosuppressive agents. *Surg. Forum* **26**, 145-146.

Kremer IB, SylvaSteenland RMR, Bos JD and Teunissen MBM (1997) Despite the presence of UVB-induced DNA damage, HLA-DR⁺ cells from *ex vivo* UVB-exposed human skin are able to migrate and show no impaired allostimulatory capacity. *J. Invest. Dermatol.* **109**, 626-631.

Kress S, Sutter C, Strickland PT, Mukhtar H, Schweizer J and Schwarz M (1992) Carcinogen-specific mutational pattern in the p53 gene in ultraviolet B radiation-induced squamous cell carcinomas of mouse skin. *Cancer Res.* **52**, 6400-6403.

Kricker A, Armstrong BK and English DR (1994) Sun exposure and non-melanocytic skin cancer. *Cancer Causes Control* **5**, 367-392.

Kricker A, Armstrong BK, English DR and Heenan PJ (1995) Does intermittent sun exposure cause basal cell carcinoma? a case-control study in Western Australia. *Int. J. Cancer* **60**, 489-494.

Kripke ML (1974) Antigenicity of murine skin tumours induced by ultraviolet light. *J. Natl. Cancer Inst.* **53**, 1333-1336.

Kripke ML and Fisher MS (1976) Immunologic parameters of ultraviolet carcinogenesis. *J. Natl. Cancer Inst.* **57**, 211-215.

Kripke ML and Fidler IJ (1980) Enhanced experimental metastasis of ultraviolet light-induced fibrosarcomas in ultraviolet light-irradiated syngeneic mice. *Cancer Res.* **40**, 625-629.

Kripke ML, Munn CG, Jeevan A, Tang JM and Bucana C (1990) Evidence that cutaneous antigen-presenting cells migrate to regional lymph nodes during contact sensitization. *J. Immunol.* **145**, 2833-2838.

Kurimoto I and Streilein JW (1992) *Cis*-urocanic acid suppression of contact hypersensitivity induction is mediated via tumor necrosis factor- α . *J. Immunol.* **148**, 3072-3078.

Kurimoto I, Kitazawa T and Streilein JW (2000) Studies of delayed systemic effects of ultraviolet B radiation (UVR) on the induction of contact hypersensitivity, 2. Evidence that interleukin-10 from UVR-treated epidermis is the critical mediator. *Immunology* **99**, 134-140.

Kutsch CL, Norris DA and Arend WP (1993) Tumor necrosis factor-alpha induces interleukin-1 alpha and interleukin-1 receptor antagonist production by cultured human keratinocytes. *J. Invest. Dermatol.* **101**, 79-85.

Laihia JK, Jansen CT, Uksila J, Punnonen J, Neuvonen K, Pasanen P and Ayras P (1994) Effects of *cis*- and *trans*-urocanic acids on the secretion of interleukin-1 beta and tumour necrosis factor-alpha by human peripheral blood monocytes. *Acta Derm. Venereol.* **74**, 266-268.

Laihia JK, Attila M, Neuvonen K, Pasanen P, Tuomisto L and Jansen CT (1998) Urocanic acid binds to GABA but not to histamine (H1, H2, or H3) receptors. *J. Invest. Dermatol.* **111**, 705-706.

Lappin MB, Weiss JM, Schopf E, Norval M and Simon JC (1997) Physiologic doses of urocanic acid do not alter the allostimulatory function or the development of murine dendritic cells *in vitro*. *Photodermatol. Photoimmunol. Photomed.* **13**, 163-168.

Lasarte JJ, Corrales FJ, Casares N, de Cerio ALD, Qian C, Xie XM, Borrás-Cuesta F and Prieto J (1999) Different doses of adenoviral vector expressing IL-12 enhance or depress the immune response to a coadministered antigen: The role of nitric oxide. *J. Immunol.* **162**, 5270-5277.

Lauerma AI, Aioi A and Maibach HI (1995) Topical *cis*-urocanic acid suppresses both induction and elicitation of contact hypersensitivity in BALB/c mice. *Acta. Derm. Venereol.* **75**, 272-275.

Ley RD (2001) Dose response for ultraviolet radiation A-induced focal melanocytic hyperplasia and nonmelanoma skin tumors in *Monodelphis domestica*. *Photochem. Photobiol.* **73**, 20-23.

Love-Schimenti CD and Kripke ML (1994) Inhibitory effect of a dendritic epidermal T cell line on K1735 melanoma cells *in vivo* and *in vitro*. *J. Leukoc. Biol.* **55**, 379-384.

Lucas AD and Halliday GM (1999) Progressor but not regressor skin tumours inhibit Langerhans' cell migration from epidermis to local lymph nodes. *Immunology* 97, 130-137.

Luscombe CJ, Fryer AA, French ME, Liu S, Saxby MF, Jones PW and Strange RC (2001) Exposure to ultraviolet radiation: association with susceptibility and age at presentation with prostate cancer. *Lancet* 358, 641-642.

Macatonia SE, Taylor PM, Knight SC and Askonas BA (1989) Primary stimulation by dendritic cells induces antiviral proliferative and cytotoxic T cell responses *in vitro*. *J. Exp. Med.* 169, 1255-1264.

MacKie RM, Bray CA, Hole DJ, Morris A, Nicolson M, Evans A, Doherty V and Vestey J; Scottish Melanoma Group (2002) Incidence of and survival from malignant melanoma in Scotland: an epidemiological study. *Lancet* 360, 587-591.

Madronich S, McKenzie RL, Bjorn LO and Caldwell MM (1998) Changes in biologically active ultraviolet radiation reaching the earth's surface. *J. Photochem. Photobiol: B-Biol.* 46, 5-19.

Manetti R, Parronchi P, Giudizi MG, Piccinni MP, Maggi E, Trinchieri G and Romagani S (1993) Natural-killer-cell stimulatory factor (Interleukin-12 (IL-12)) induces T-helper type-1 (Th1)-specific immune responses and inhibits the development of IL-4 producing Th cells. *J. Exp. Med.* 177, 1199-1204.

Marks R, Foley P, Goodman G, Hage BH and Selwood TS (1986) Spontaneous remission of solar keratoses: the case for conservative management. *Br. J. Dermatol.* 115, 649-655.

Marshall V (1974) Premalignant and malignant skin tumours in immunosuppressed patients. *Transplantation* 17, 272-275.

Matheson MJ and Reeve VE (1991) The effect of the antihistamine cimetidine on ultraviolet-radiation-induced tumorigenesis in the hairless mouse. *Photochem. Photobiol.* **53**, 639-642.

McBride WH (1986) Phenotype and functions of intratumoral macrophages. *Biochim. Biophys. Acta* **865**, 27-41.

McBride WH and Howie SEM (1986) Induction of tolerance to a murine fibrosarcoma in two zones of dosage – the involvement of suppressor cells. *Brit. J. Cancer* **53**, 707-711.

McBride WH, Thacker JD, Comora S, Economou JS, Kelley D, Hogge D, Dubinett SM and Dougherty GJ (1992) Genetic modification of a murine fibrosarcoma to produce interleukin-7 stimulates host cell infiltration and tumour immunity. *Cancer Res.* **52**, 2931-3937.

McKenzie RL, Bjorn LO, Bais A and Ilyas M (2003) Changes in biologically active ultraviolet radiation reaching the Earth's surface. *Photochem. Photobiol. Sci.* **2**, 5-15.

Meko JB, Tsung K and Norton JA (1996) Cytokine production and antitumor effect of a nonreplicating, noncytopathic recombinant vaccinia virus expressing interleukin-12. *Surgery* **120**, 274-283.

Miescher S, Whiteside TL, Carrel S and von Fliedner V (1986) Functional properties of tumor-infiltrating and blood lymphocytes in patients with solid tumors: effects of tumor cells and their supernatants on proliferative responses of lymphocytes. *J. Immunol.* **136**, 1899-1907.

Milas L, Hunter N, Mason K and Withers HR (1974) Immunological resistance to pulmonary metastases in C3Hf-Bu mice bearing syngeneic fibrosarcoma of different sizes. *Cancer Res.* **34**, 61-71.

- Miyauchi H and Horio Y (1995) Ultraviolet B-induced local immunosuppression of contact hypersensitivity is modulated by ultraviolet irradiation and hapten application. *J. Invest. Dermatol.* **104**, 364-369.
- Moan J and Dahlback A (1992) The relationship between skin cancers, solar radiation and ozone depletion. *Br. J. Cancer* **65**, 916-921.
- Moodycliffe AM, Kimber I and Norval M (1992) The effect of ultraviolet B radiation and urocanic acid isomers on dendritic cell migration. *Immunology* **77**, 394-399.
- Moodycliffe AM, Norval M, Kimber I and Simpson TJ (1993) Characterization of a monoclonal antibody to *cis*-urocanic acid: detection of *cis*-urocanic acid in the serum of irradiated mice by immunoassay. *Immunology* **79**, 667-672.
- Moodycliffe AM, Kimber I and Norval M (1994) Role of tumour necrosis factor- α in ultraviolet B light-induced dendritic cell migration and suppression of contact hypersensitivity. *Immunology* **81**, 79-84.
- Moodycliffe AM, Bucana CD, Kripke ML, Norval M and Ullrich SE (1996) Differential effects of a monoclonal antibody to *cis*-urocanic acid on the suppression of delayed and contact hypersensitivity following ultraviolet irradiation. *J. Immunol.* **157**, 2891-2899.
- Moodycliffe AM, Nghiem D, Clydesdale G and Ullrich SE (2000) Immune suppression and skin cancer development: regulation by NKT cells. *Nat. Immunol.* **1**, 521-525.
- Morison WL (1984) The effect of a sunscreen containing para-aminobenzoic acid on the systemic immunologic alterations induced in mice by exposure to UVB radiation. *J. Invest. Dermatol.* **83**, 405-408.

Müller G, Saloga J, Germann T, Schuler G, Knop J and Enk AH (1995). IL-12 as mediator and adjuvant for the induction of contact sensitivity *in vivo*. *J. Immunol.* **155**, 4661-4668.

Muller HK, Halliday GM and Knight BA (1985) Carcinogen-induced depletion of cutaneous Langerhans cells. *Br. J. Cancer* **52**, 81-85.

Mumberg D, Monach PA, Wanderling S, Philip M, Toledano AY, Schreiber RD and Schreiber H (1999) CD4(+) T cells eliminate MHC class II-negative cancer cells *in vivo* by indirect effects of IFN-gamma. *Proc. Natl. Acad. Sci. USA* **96**, 8633-8638.

Narvaez D, Kanitakis J and Claudy A (1995) Dendritic cells of human dermis. *Eur. J. Dermatol.* **5**, 69-76.

Nastala CL, Edington JD, McKinney TG, Tahara H, Nalesnik MA, Brunda MJ, Gately MK, Wolf SF, Schreiber RD, Storkus WJ *et al* (1994) Recombinant IL-12 administration induces tumour regression in association with IFN-gamma production. *J. Immunol.* **153**, 1697-1706.

Naylor MF, Boyd A, Smith DW, Cameron GS, Hubbard D and Neldner KH (1995) High sun protection factor sunscreens in the suppression of actinic neoplasia. *Arch. Dermatol.* **131**, 170-175.

Nestle FO, Zheng XG, Thompson CB, Turka LA and Nickoloff BJ (1993) Characterization of dermal dendritic cells obtained from normal human skin reveals phenotypic and functionally distinctive subsets. *J. Immunol.* **151**, 6535-6545.

Nestle FO, Alijagic S, Gilliet M, Sun Y, Grabbe S, Dummer R, Burg G and Schadendorf D (1998) Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat. Med.* **4**, 328-332.

- Nishigori C, Yarosh DB, Ullrich SE, Vink AA, Bucana CD, Roza L and Kripke ML (1996) Evidence that DNA damage triggers interleukin 10 cytokine production in UV-irradiated murine keratinocytes. *Proc. Natl. Acad. Sci. USA* **93**, 10354-10359.
- Nishigori C, Yarosh D, O'Connor A, Shreedhar VK, Ullrich SE, Cox P and Kripke ML (1998) Hind III liposomes suppress delayed-type hypersensitivity responses *in vivo* and induce epidermal IL-10 *in vitro*. *J. Immunol.* **161**, 2684-2691.
- Noonan FP, Kripke ML, Pedersen GM and Greene MI (1981) Suppression of contact hypersensitivity in mice by ultraviolet irradiation is associated with defective antigen presentation. *Immunology* **43**, 527-533.
- Noonan FP, Bucana C, Sauder DN and De Fabo EC (1984) Mechanism of systemic immune suppression by UV irradiation *in vivo*. II. The UV effects on number and morphology of epidermal Langerhans cells and the UV-induced suppression of contact hypersensitivity have different wavelength dependencies. *J. Immunol.* **132**, 2408-2416.
- Noonan FP, De Fabo EC and Morrison H (1988) *Cis*-urocanic acid, a product formed by ultraviolet B irradiation of the skin, initiates an antigen presentation defect in splenic dendritic cells *in vivo*. *J. Invest. Dermatol.* **90**, 92-99.
- Noonan FP, Recio JA, Takayama H, Duray P, Anver MR, Rush WL, De Fabo EC and Merlino G (2001) Neonatal sunburn and melanoma in mice. *Nature* **413**, 271-272.
- Norval M, McIntyre CR, Simpson TJ, Howie SE and Bardshiri E (1988) Quantification of urocanic acid isomers in murine skin during development and after irradiation with UVB light. *Photodermatol.* **5**, 179-186.
- Norval M, Simpson TJ, Bardshiri E and Howie SE (1989) Urocanic acid analogues and the suppression of the delayed type hypersensitivity response to Herpes simplex virus. *Photochem. Photobiol.* **49**, 633-639.

- Petit-Frere C, Clingen PH, Grewe M, Krutmann J, Roza L, Arlett CF and Green MH (1998) Induction of interleukin-6 production by ultraviolet radiation in normal human epidermal keratinocytes and in a human keratinocyte cell line is mediated by DNA damage. *J. Invest. Dermatol.* **111**, 354-359.
- Porgador A, Snyder D and Gilboa E (1996) Induction of antitumor immunity using bone marrow-generated dendritic cells. *J. Immunol.* **156**, 2918-2926.
- Prechel MM, Lozano Y, Wright MA, Ihm J and Young MRI (1995) Ineffective immune enhancement by IL-12 in tumour-bearing mice whose immune depression is mediated by suppressive granulocyte-macrophage progenitor cells. *Cancer Lett.* **92**, 235-242.
- Raasch BA and Buettner PG (2002) Multiple nonmelanoma skin cancer in an exposed Australian population. *Int. J. Dermatol.* **41**, 652-658.
- Rady P, Scinicariello F, Wagner RF Jr and Tyring SK (1992) p53 mutations in basal cell carcinomas. *Cancer Res.* **52**, 3804-3806.
- Rasanen L, Reunala T, Lehto M, Jansen C, Rantala I and Leinikki P (1989) Immediate decrease in antigen-presenting function and delayed enhancement of interleukin-1 production in human epidermal cells after *in vivo* UVB irradiation. *Br. J. Dermatol.* **120**, 589-596.
- Rattis FM, Peguet-Navarro J, Courtellemont P, Redziniak G and Schmitt D (1995) *Cis*-urocanic acid failed to affect *in vitro* human Langerhans cell allostimulatory function. *Photochem. Photobiol.* **62**, 914-916.
- Rattis FM, Concha M, Dalbiez-Gauthier C, Courtellemont P and Peguet-Navarro J (1998) Effects of ultraviolet B radiation on human Langerhans cells: Functional alteration of CD86 upregulation and induction of apoptotic cell death. *J. Invest. Dermatol.* **111**, 373-379.

Norval M, Gilmour JW and Simpson TJ (1990) The effect of histamine receptor antagonists on immunosuppression induced by the *cis*-isomer of urocanic acid. *Photodermatol. Photoimmunol. Photomed.* **7**, 243-248.

Obata M and Tagami H (1985) Alteration in murine epidermal Langerhans cell population by various UV irradiations: quantitative and morphologic studies on the effects of various wavelengths of monochromatic radiation on Ia-bearing cells. *J. Invest. Dermatol.* **84**, 139-145.

O'Connor A, Nishigori C, Yarosh D, Alas L, Kibitel J, Burley L, Cox P, Bucana C, Ullrich S and Kripke M (1996) DNA double strand breaks in epidermal cells cause immune suppression *in vivo* and cytokine production *in vitro*. *J. Immunol.* **157**, 271-278.

Ohmen JD, Moy RL, Zovich D, Lieberman A, Wyzykowski RJ, Sullivan L, Modlin RL and Uyemura K (1994) Selective accumulation of T cells according to T-cell receptor V beta gene usage in skin cancer. *J. Invest. Dermatol.* **103**, 751-757.

Okamoto H, Itoh K, Welsh E, Trial J, Platsoucas C, Bucana C and Kripke ML (1988) *In vitro* cytotoxic activity of interleukin 2-dependent murine Thy-1⁺ dendritic epidermal cell lines. *J. Leukoc. Biol.* **43**, 502-508.

Okamoto H, Mizuno K, Itoh T, Tanaka K and Horio T (1999) Evaluation of apoptotic cells induced by ultraviolet light B radiation in epidermal sheets stained by the TUNEL technique. *J. Invest. Dermatol.* **113**, 802-807.

Pardoll DM (1994) Tumour antigens. A new look for the 1990s. *Nature* **369**, 357.

Pavey S, Conroy S, Russell T and Gabrielli B (1999) Ultraviolet radiation induces p16CDKN2A expression in human skin. *Cancer Res.* **59**, 4185-4189.

Redondo P, Garcia-Foncillas J, Cuevillas F, Espana A and Quintanilla E (1996) Effects of low concentrations of *cis*- and *trans*-urocanic acid on cytokine elaboration by keratinocytes. *Photodermatol. Photoimmunol. Photomed.* **12**, 237-243.

Reeve VE, Greenoak GE, Canfield PJ, Boehm-Wilcox C and Gallagher CH (1989) Topical urocanic acid enhances UV-induced tumour yield and malignancy in the hairless mouse. *Photochem. Photobiol.* **49**, 459-464.

Reeve VE, Matheson MJ, Bosnic M and Boehm-Wilcox C (1995) The protective effect of indomethacin on photocarcinogenesis in hairless mice. *Cancer Lett.* **95**, 213-219.

Rivas JM and Ullrich SE (1992) Systemic suppression of delayed-type hypersensitivity by supernatants from UV-irradiated keratinocytes. An essential role for keratinocyte-derived IL-10. *J. Immunol.* **149**, 3865-3871.

Rizos H, Darmanian AP, Indsto JO, Shannon JA, Kefford RF and Mann GJ (1999) Multiple abnormalities of the p16INK4a-pRb regulatory pathway in cultured melanoma cells. *Melanoma Res.* **9**, 10-19.

Roberts LK (1986) Characterization of a cloned ultraviolet radiation (UV)-induced suppressor T cell line that is capable of inhibiting anti-UV tumor-immune responses. *J. Immunol.* **136**, 1908-1916.

Roberts LK and Beasley DG (1997) Sunscreen lotions prevent ultraviolet radiation-induced suppression of antitumor immune responses. *Int. J. Cancer* **71**, 94-102.

Robinson ES, Hubbard GB, Colon G and Vandeberg JL (1998) Low-dose ultraviolet exposure early in development can lead to widespread melanoma in the opossum model. *Int. J. Exp. Pathol.* **79**, 235-244.

Romerdaal CA, Donawho C, Fidler IJ and Kripke ML (1988) Effect of ultraviolet B radiation on the *in vivo* growth of murine melanoma cells. *Cancer Res.* **48**, 4007-4010.

RomerdaHL CA and Kripke ML (1988) Role of helper T-lymphocytes in rejection of UV-induced murine skin cancers. *Cancer Res.* **48**, 2325-2328.

Ross JA, Howie SE, Norval M, Maingay J, and Simpson TJ (1986) Ultraviolet-irradiated urocanic acid suppresses delayed-type hypersensitivity to herpes simplex virus in mice. *J. Invest. Dermatol.* **87**, 630-633.

Rosso S, Zanetti R, Martinez C, Tormo MJ, Schraub S, Sancho-Garnier H, Franceschi S, Gafa L, Perea E, Navarro C, Laurent R, Schrameck C, Talamini R, Tumino R and Wechsler J (1996) The multicentre south European study 'Helios'. II: Different sun exposure patterns in the aetiology of basal cell and squamous cell carcinomas of the skin. *Br. J. Cancer* **73**, 1447-1454.

Ruedl C, Koebel P, Bachmann M, Hess M and Karjalainen K (2000) Anatomical origin of dendritic cells determines their life span in peripheral lymph nodes. *J. Immunol.* **165**, 4910-4916.

Ryffel B (1997) Interleukin-12: role of interferon-gamma in IL-12 adverse effects. *Clin. Immunol. Immunopathol.* **83**, 1-4.

Saijo S, Bucana CD, Ramirez KM, Cox PA, Kripke ML and Strickland FM (1995) Deficient antigen presentation and Ts induction are separate effects of ultraviolet irradiation. *Cell Immunol.* **164**, 189-202.

Schmitt DA, Owenschaub L and Ullrich SE (1995) Effect of IL-12 on immune suppression and suppressor cell induction by ultraviolet radiation. *J. Immunol.* **154**, 5114-5120.

Schmitt DA, Walterscheid JP and Ullrich SE (2000) Reversal of ultraviolet radiation-induced immune suppression by recombinant interleukin-12; suppression of cytokine production. *Immunology* **101**, 90-96.

Schuhmachers G, Ariizumi K, Mathew PA, Bennett M, Kumar V and Takashima A (1995) 2B4, a new member of the immunoglobulin gene superfamily, is expressed on murine dendritic epidermal T cells and plays a functional role in their killing of skin tumors. *J. Invest. Dermatol.* **105**, 592-596.

Schwarz A, Grabbe S, Aragane Y, Sandkuhl K, Riemann H, Luger TA, Kubin M, Trinchieri G and Schwarz T (1996) Interleukin-12 prevents ultraviolet B-induced local immunosuppression and overcomes UVB-induced tolerance. *J. Invest. Derm.* **6**, 1187-1191.

Schwarz A, Beissert S, Grosse-Heitmeyer K, Gunzer M, Bluestone JA, Grabbe S and Schwarz T (2000) Evidence for functional relevance of CTLA-4 in ultraviolet-radiation-induced tolerance. *J. Immunol.* **165**, 1824-1831.

Schwarz A, Ständer S, Berneburg M, Böhm M, Kulms D, van Steeg H, Grosse-Heitmeyer K, Krutmann J and Schwarz T (2002) Interleukin-12 suppresses ultraviolet radiation-induced apoptosis by inducing DNA repair. *Nat. Cell Biol.* **4**, 26-31.

Scott IR (1981) Factors controlling the expressed activity of histidine ammonia-lyase in the epidermis and the resulting accumulation of urocanic acid. *Biochem. J.* **194**, 829-838.

Scott IR, Harding CR and Barrett JG (1982) Histidine-rich protein of the keratohyalin granules. Source of the free amino acids, urocanic acid and pyrrolidone carboxylic acid in the stratum corneum. *Biochim. Biophys. Acta* **719**, 110-117.

Sharma S, Stolina M, Lin Y, Gardner B, Miller PW, Kronenberg M and Dubinett SM (1999) T cell-derived IL-10 promotes lung cancer growth by suppressing both T cell and APC function. *J. Immunol.* **163**, 5020-5028.

Shen J, Bao S and Reeve VE (1999) Modulation of IL-10, IL-12, and IFN-gamma in the epidermis of hairless mice by UVA (320-400 nm) and UVB (280-320 nm) radiation. *J. Invest. Dermatol.* **113**, 1059-1064.

Shimizu T and Streilein JW (1994a) Local and systemic consequences of acute, low-dose ultraviolet B radiation are mediated by different immune regulatory mechanisms. *Eur. J. Immunol.* **54**, 1765-1770.

Shimizu T and Streilein JW (1994b) Evidence that ultraviolet B radiation induces tolerance and impairs induction of contact hypersensitivity by different mechanisms. *Immunology* **82**, 140-148.

Shreedhar VK, Pride MW, Sun Y, Kripke ML and Strickland FM (1998) Origin and characteristics of ultraviolet-B radiation-induced suppressor T lymphocytes. *J. Immunol.* **161**, 1327-1335.

Simon JC, Cruz PD Jr, Bergstresser PR and Tigelaar RE (1990) Low dose ultraviolet B-irradiated Langerhans cells preferentially activate CD4⁺ cells of the T helper 2 subset. *J. Immunol.* **145**, 2087-2091.

Skov L, Hansen H, Dittmar HC, Barker JNWN, Simon JC and Baadsgaard O (1998) Susceptibility to effects of UVB irradiation on induction of contact sensitivity, relevance of number and function of Langerhans cells and epidermal macrophages. *Photochem. Photobiol.* **67**, 714-719.

Sluyter R and Halliday GM (2000) Enhanced tumor growth in UV-irradiated skin is associated with an influx of inflammatory cells into the epidermis. *Carcinogenesis* **21**, 1801-1807.

Sluyter R, Yuen KS and Halliday GM (2001) Protective immunity to UV radiation-induced skin tumours induced by skin grafts and epidermal cells. *Immunol. Cell Biol.* **79**, 29-34.

Smith JB, McIntosh GH and Morris B (1970) The traffic of cells through tissues: a study of peripheral lymph in sheep. *J. Anat.* **107**, 87-100.

Smolle J, Soyer HP, Ehall R, Bartenstein S and Kerl H (1986) Langerhans cell density in epithelial skin tumors correlates with epithelial differentiation but not with the peritumoral infiltrate. *J. Invest. Dermatol.* **87**, 477-479.

Snellman E, Jansen CT, Rantanen T and Pasanen P (1999) Epidermal urocanic acid concentration and photoisomerization reactivity in patients with cutaneous malignant melanoma or basal cell carcinoma. *Acta Derm. Venereol.* **79**, 200-203.

Sontag Y, Guikers CLH, Vink AA, de Gruijl FR, Van Loveren H, Garssen J, Roza L, Kripke ML, van der Leun JC and van Vloten WA (1995) Cells with UV-specific DNA damage are present in murine lymph nodes after *in vivo* UV irradiation. *J. Invest. Dermatol.* **104**, 734-738.

Steerenberg PA, De Jong WH, Geerse E, De Graaf A, Scheper RJ, Den Otter W and Ruitenbergh EJ (1991) Major histocompatibility complex class II antigen expression on leucocyte subpopulations in the draining lymph node and tumour in the early phase of bacillus-Calmette-Guerin-induced tumour regression. *Cancer Immunol. Immunother.* **33**, 189-197.

Stingl LA, Sauder DN, Iijima M, Wolff K, Pehamberger H and Stingl G (1983) Mechanism of UV-B-induced impairment of the antigen-presenting capacity of murine epidermal cells. *J. Immunol.* **130**, 1586-1591.

Streilein JW and Bergstresser PR (1988) Genetic basis of ultraviolet-B effects on contact hypersensitivity. *Immunogenetics* **27**, 252-258.

Strickland FM, Sun Y, Darvill A, Eberhard S, Pauly M and Albersheim P (2001) Preservation of the delayed-type hypersensitivity response to alloantigen by xyloglucans or oligogalacturonide does not correlate with the capacity to reject ultraviolet-induced skin tumors in mice. *J. Invest. Dermatol.* **116**, (62-68).

Suit HD and Kastellan A (1970) Immunologic status of host and response to a methylcholanthrene-induced fibrosarcoma to local irradiation. *Cancer* **26**, 232-238.

Swerdlow (1985) Seasonality of presentation of cutaneous melanoma, squamous cell cancer and basal cell cancer in the Oxford region. *Br. J. Cancer* **52**, 893-900.

Synkowski DR, Schuster P and Orlando JC (1985) The immunobiology of basal cell carcinoma: an in situ monoclonal antibody study. *Br. J. Dermatol.* **113**, 441-446.

Tang A and Udey MC (1991) Inhibition of epidermal Langerhans cell function by low dose ultraviolet B radiation. Ultraviolet B radiation selectively modulates ICAM-1 (CD54) expression by murine Langerhans cells. *J. Immunol.* **146**, 3347-3355.

Tang A and Udey MC (1992) Effects of ultraviolet-radiation on murine epidermal Langerhans cells - doses of ultraviolet-radiation that modulate ICAM-1 (CD54) expression and inhibit Langerhans cell function cause delayed cytotoxicity *in vitro*. *J. Invest. Dermatol.* **99**, 83-89.

Tefany FJ, Barnetson RS, Halliday GM, McCarthy SW and McCarthy WH (1991) Immunocytochemical analysis of the cellular infiltrate in primary regressing and non-regressing malignant melanoma. *J. Invest. Dermatol.* **97**, 197-202.

Teunissen MB, Rongen HA and Bos JD (1994) Function of adhesion molecules lymphocyte function-associated antigen-3 and intercellular adhesion molecule-1 on human epidermal Langerhans cells in antigen-specific T cell activation. *J. Immunol.* **152**, 3400-3409.

Teunissen MB, Koomen CW, Jansen J, de Waal Malefyt R, Schmitt E, Van den Wijngaard RM, Das PK and Bos JD (1997) In contrast to their murine counterparts, normal human keratinocytes and human epidermoid cell lines A431 and HaCaT fail to express IL-10 mRNA and protein. *Clin. Exp. Immunol.* **107**, 213-223.

Teunissen MB, Piskin G, di Nuzzo S, Sylva-Steenland RM, de Rie MA and Bos JD (2002) Ultraviolet B radiation induces a transient appearance of IL-4⁺ neutrophils, which support the development of Th2 responses. *J. Immunol.* **168**, 3732-3739.

Thompson SC, Jolley D and Marks R (1993) Reduction of solar keratoses by regular sunscreen use. *N. Engl. J. Med.* **329**, 1147-1151.

Toda K, Miyachi Y, Kuribayashi K and Imamura S (1986) Decreased NK activity of nude mice receiving long-term ultraviolet irradiation: in relation to tumor induction. *J. Clin. Lab. Immunol.* **20**, 129-131.

Toews GB, Bergstresser PR and Streilein JW (1980) Epidermal Langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB. *J. Immunol.* **124**, 445-453.

Townley SL, Grimbaldston MA, Ferguson I, Rush RA, Zhang SH, Zhou XF, Conner JM, Finlay-Jones JJ and Hart PH (2002) Nerve growth factor, neuropeptides, and mast cells in ultraviolet-B-induced systemic suppression of contact hypersensitivity responses in mice. *J. Invest. Dermatol.* **118**, 396-401.

Trinchieri G, Kubin M, Bellone G and Cassatella MA (1993) Cytokine cross-talk between phagocytic cells and lymphocytes – relevance for differentiation activation of phagocytic cells and regulation of adaptive immunity. *J. Cell. Biochem.* **53**, 310-308

Tse Y and Cooper KD (1990) Cutaneous dermal Ia⁺ cells are capable of initiating delayed type hypersensitivity responses. *J. Invest. Dermatol.* **94**, 267-272.

Tsoukas CD, Landgraf B, Bentin J, Valentine M, Lotz M, Vaughan JH and Carson DA (1985) Activation of resting T lymphocytes by anti-CD3 (T3) antibodies in the absence of monocytes. *J. Immunol.* 135, 1719-1723.

Uksila J, Laihia JK and Jansen CT (1994) *Trans*-urocanic acid, a natural epidermal constituent, inhibits human natural killer cell activity *in vitro*. *Exp. Dermatol.* 3, 61-65.

Ullrich SE (1995) The role of epidermal cytokines in the generation of cutaneous immune reactions and ultraviolet radiation-induced immune suppression. *Photochem. Photobiol.* 62, 389-401.

Van der Hove LE, Van Gool SW, Van Poppel H, Baert L, Coorevits L, Van Damme B and Ceuppens JL (1997) Phenotype, cytokine production and cytolytic capacity of fresh (uncultured) tumour-infiltrating T lymphocytes in human renal cell carcinoma. *Clin. Exp. Immunol.* 109, 501-509.

Vermes I, Haanen C, Steffens-Nakken H and Reutelingsperger C (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods* 184, 39-51.

Vink AA, Strickland FM, Bucana C, Cox PA, Roza L, Yarosh DB and Kripke ML (1996) Localization of DNA damage and its role in altered antigen-presenting cell function in ultraviolet-irradiated mice. *J. Exp. Med.* 183, 1491-1500.

Vink AA, Moodycliffe AM, Shreedhar V, Ullrich SE, Roza L, Yarosh DB and Kripke ML (1997) The inhibition of antigen-presenting activity of dendritic cells resulting from UV irradiation of murine skin is restored by *in vitro* photorepair of cyclobutane pyrimidine dimers. *Proc. Natl. Acad. Sci. USA* 94, 5255-5260.

Vitasa BC, Taylor HR, Strickland PT, Rosenthal FS, West S, Abbey H, Ng SK, Munoz B and Emmett EA (1990) Association of nonmelanoma skin cancer and actinic keratosis with cumulative solar ultraviolet exposure in Maryland watermen. *Cancer* **65**, 2811-2817.

Walterscheid JP, Ullrich SE and Nghiem DX (2002) Platelet-activating factor, a molecular sensor for cellular damage, activates systemic immune suppression. *J. Invest. Dermatol.* **195**, 171-179.

Walunas TL, Bakker CY and Bluestone JA (1996) CTLA-4 ligation blocks CD28-dependent T cell activation. *J. Exp. Med.* **183**, 2541-2550.

Ward PL, Koeppen H, Hurteau T and Schreiber H (1989) Tumor antigens defined by cloned immunological probes are highly polymorphic and are not detected on autologous normal cells. *J. Exp. Med.* **170**, 217-232.

Weichenthal M, Godorr M, Altenhoff J, Neuber K and Breitbart EW (2000) Effects of whole-body UVB irradiation on cytokine production by peripheral blood mononuclear cells from stage I melanoma patients. *Arch. Dermatol. Res.* **292**, 348-353.

Weimar VM, Ceilley RI and Goeken JA (1979) Aggressive biologic behavior of basal- and squamous-cell cancers in patients with chronic lymphocytic leukemia or chronic lymphocytic lymphoma. *J. Dermatol. Surg. Oncol.* **5**, 609-614.

Whiteman DC, Whiteman CA and Green AC (2001) Childhood sun exposure as a risk factor for melanoma: a systematic review of epidemiologic studies. *Cancer Causes Control* **12**, 69-82.

Wikner NE, Huff JC, Norris DA, Boyce ST, Cary M, Kissinger M and Weston WL (1986) Study of HLA-DR synthesis in cultured human keratinocytes. *J. Invest. Dermatol.* **87**, 559-564.

Wille JJ, Kydonieus AF and Murphy GF (1999) *Cis*-urocanic acid induces mast cell degranulation and release of preformed TNF- α : A possible mechanism linking UVB and *cis*-urocanic acid to immunosuppression of contact hypersensitivity. *Skin Pharmacol. Appl. Skin Physiol.* **12**, 18-27.

Wiltshcke C, Krainer M, Budinsky AC, Berger A, Muller C, Zeillinger R, Speiser P, Kubista E, Eibl M, and Zielinski CC (1995) Reduced mitogenic stimulation of peripheral blood mononuclear cells as a prognostic parameter for the course of breast cancer: a prospective longitudinal study. *Br. J. Cancer* **71**, 1292-1296.

Wolf P, Donawho CK and Kripke ML (1994) Effect of sunscreens on UV radiation-induced enhancement of melanoma growth in mice. *J. Natl. Cancer Inst.* **86**, 99-105.

Wolf P, Cox P, Yarosh DB and Kripke ML (1995) Sunscreens and T4N5 liposomes differ in their ability to protect against ultraviolet-induced sunburn cell formation, alterations of dendritic epidermal cells, and local suppression of contact hypersensitivity. *J. Invest. Dermatol.* **104**, 287-292.

Won Byeon S, Pelly RP, Ullrich SE, Waller TA, Bucana CD and Strickland FM (1998) Aloe barbadensis extracts reduce the production of interleukin-10 after exposure to ultraviolet radiation. *J. Invest. Dermatol.* **110**, 811-817.

Yamaji K, Matsui M and Saida T (1987) Increased densities of Langerhans cells in the epidermis of skin tumors. *J. Dermatol.* **14**, 20-24.

Yarosh D, Klein J, O'Connor A, Hawk J, Rafal E and Wolf P (2001) Effect of topically applied T4 endonuclease V in liposomes on skin cancer in xeroderma pigmentosum: a randomised study. *Lancet* **357**, 926-929.

Yawalkar N, Aebischer MC, Hunger R, Brand CU and Braathen LR (1998) Effects of UV irradiation with one minimal erythema dose on human afferent skin lymph *in vivo*. *Exp. Dermatol.* **7**, 362-368.

Yoshikawa T, Rae V, Bruins-Slot W, Van den Berg JW, Taylor JR and Streilein JW (1990) Susceptibility to effects of UVB radiation on induction of contact hypersensitivity as a risk factor for skin cancer in humans. *J. Invest. Dermatol.* **95**, 530-536.

Yoshizawa H, Chang AE and Shu SY (1992) Cellular interactions in effector cell generation and tumor regression mediated by anti-CD3/interleukin 2-activated tumor-draining lymph node cells. *Cancer Res.* **52**, 1129-1136.

Zak-Prelich M, Norval M, Venner TJ, Bisset Y, Walker C, Rafferty TS, Sauder DN and McKenzie RC (2001) *Cis*-Urocanic acid does not induce the expression of immunosuppressive cytokines in murine keratinocytes. *Photochem. Photobiol.* **73**, 238-244.

Zhang XD, Schiller GD, Gill PG and Coventry BJ (1998) Lymphoid cell infiltration during breast cancer growth: a syngeneic rat model. *Immunol. Cell Biol.* **76**, 550-555.

Ziegler A, Leffell DJ, Kunala S, Sharma HW, Gailani M, Simon JA, Halperin AJ, Baden HP, Shapiro PE, Bale AE, *et al* (1993) Mutation hotspots due to sunlight in the p53 gene of nonmelanoma skin cancers. *Proc. Natl. Acad. Sci. USA* **90**, 4216-4220.

Ziegler A, Jonason AS, Leffell DJ, Simon JA, Sharma HW, Kimmelman J, Remington L, Jacks T and Brash DE (1994) Sunburn and p53 in the onset of skin cancer. *Nature* **372**, 773-776.

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The effects of UV waveband and *cis*-urocanic acid on tumour outgrowth in mice

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Immunogenic murine tumours that are normally rejected upon transplantation into syngeneic hosts grow progressively if the hosts are UV-irradiated prior to tumour implantation. Using three sources of UV we investigated the most effective waveband and dose for increased outgrowth of injected fibrosarcoma (FSA) cells in mice, compared with unirradiated controls. The animals were exposed on their shaved dorsal skin twice a week for 3 weeks to either broad-band (TL12) or narrow-band (TL01) UVB, or UVA-I radiation; FSA cells were then injected subcutaneously into the UV-irradiated skin. Exposure to TL12 at doses higher than 1000 J m^{-2} , but not TL01 or UVA-I, caused increased outgrowth of the injected tumour cells. The effect of the UV irradiation seemed to be local as injection of the FSA cells into the unirradiated ventral skin of mice irradiated with TL12 on the dorsal surface did not result in increased outgrowth of the tumours. *Cis*-urocanic acid, a recognised initiator of UV-induced immunosuppression, applied topically or intradermally for 3 weeks prior to FSA cell injection, had no effect on the rate of tumour outgrowth. Similarly, injection of a monoclonal antibody with specificity for *cis*-urocanic acid, prior to each irradiation with TL12, did not reverse the increased growth of FSA cells injected into the UV-irradiated skin. Thus wavelengths within the broad-band UVB range are the most effective for inducing increased outgrowth of FSA cells; *cis*-urocanic acid is not an important mediator in this UV-enhanced growth of tumours.

Introduction

Skin tumours, induced in mice by exposure to ultraviolet radiation (UVR) are mostly strongly antigenic, and do not grow when transplanted into normal syngeneic hosts.¹ However prior exposure of the recipient mice to UVR twice a week for 4 weeks, results in progressive growth of subsequently transplanted antigenic tumours.² This effect on tumour outgrowth can be transferred to unirradiated mice by spleen cells from UV-irradiated mice, suggesting that UV exposure suppresses the immune response to these immunogenic skin tumours.³ The effect of different UV wavelengths on outgrowth of implanted tumours was investigated by De Fabo and Kripke,⁴ using a fibrosarcoma (FSA) tumour that does not grow progressively upon transplantation into normal, syngeneic mice. Exposure of mice to wavelengths between 255–340 nm prior to tumour implantation resulted in progressive growth of the implanted tumours; removal of wavelengths below 275 nm did not alter this result. Mice exposed to wavelengths above 315 nm prior to tumour implantation, were not susceptible to tumour challenge; the most important waveband was therefore in the range 275–315 nm.

A study using Skh-1 albino hairless mice demonstrated that the most effective wavelength for induction of skin cancer was 293 nm, which lies within the UVB (290–320 nm) range.⁵ A shoulder of effectiveness at 10^{-4} of the maximum resulted from exposure of mice to wavelengths over 340 nm, suggesting a possible role for UVA (320–400 nm) in the generation of skin cancer.

There has been much investigation into the immunosuppressive properties of UV, especially UVB, which suppresses both contact hypersensitivity (CH) and delayed type hypersensitivity (DTH); these have been used as models for UVB-induced suppression of T-cell responses. Following UVB exposure, there are changes in cytokine profiles (reviewed in ref. 6), alterations in the number⁷ and morphology⁸ of epidermal antigen presenting cells (APCs), and induction of regulatory

cells in the spleen and lymph nodes.^{9,10} Wavelengths within the UVB range do not penetrate past the upper layer of the dermis; therefore it is thought that UV initiates suppression through a photoreceptor in the skin. Two photoreceptors with major roles in UV-induced immunosuppression are DNA¹¹ and urocanic acid.¹² Urocanic acid (UCA), which is found in the skin as the *trans* isomer, is converted by UVR to the *cis* isomer; *cis*-UCA has been demonstrated to suppress both CH and DTH responses, and to affect the function of APCs (reviewed in ref. 13).

Interest in the effects of wavelengths in the UVA range on immune responses has grown in recent years. Prior irradiation of mice with UVA waveband has been shown to protect against UVB-induced suppression of CH responses.¹⁴ On the other hand, UVA exposure alone can suppress both CH and DTH responses.¹⁵ The mechanism is likely to be different from that occurring as a result of UVB exposure and is probably through the production of reactive oxygen species.¹⁶

In the present study mice were irradiated for a period of 3 weeks; cells from a moderately immunogenic fibrosarcoma were then injected subcutaneously, and outgrowth of the subsequent tumours measured. The effects of broad-band (280–360 nm) and narrowband (311–313 nm) UVB, and UVA-I (340–400 nm) on the outgrowth of the FSA cells were compared; the role of *cis*-UCA in UV-enhanced tumour outgrowth was also investigated.

Materials and Methods

Mice

Female C3H/HeN (H-2k) mice aged 6–10 weeks were obtained from the Medical Faculty Animal Area, University of Edinburgh; they were housed in a room where ambient light was regulated on a 12 hour light/dark cycle, and had free access to food and water. Room lights were shielded such that any contaminating UV wavelengths were filtered out. All experi-

ments were performed according to the ethical guidelines of the University of Edinburgh and the Home Office.

Tumour cells

The fibrosarcoma (FSA) cell line was a gift from Professor William McBride (University of California, Los Angeles). The cells are moderately immunogenic and are derived from a fibrosarcoma that was induced in C3H mice by methylcholanthrene treatment.¹⁷ The tumours formed after subcutaneous injection have been well characterised with respect to the immune response generated, including the intratumoural host cell population, consisting mainly of macrophages with a small component of CD4 and CD8 positive cells.¹⁸ Cells were stored in liquid N₂ and, prior to injection into mice, were cultured in RPMI (Gibco, BRL, Paisley, UK) supplemented with 100 i.u. ml⁻¹ penicillin, 200 µg ml⁻¹ streptomycin, 2 mM L-glutamine, 100 µg ml⁻¹ gentamicin, 1.5 µg ml⁻¹ fungizone and 10% heat-inactivated foetal calf serum; cells were passaged a maximum of three times prior to injection into mice. Subconfluent monolayers were harvested using a 0.1% trypsin/0.04% versene solution and the number of viable cells determined by trypan blue exclusion. For injection into mice, cells were washed and resuspended in Hanks' balanced salt solution (Gibco, BRL), supplemented with 20 µg ml⁻¹ deoxyribonuclease I (Sigma, Poole, Dorset, UK). Mice were anaesthetised with halothane 24 h after the final irradiation and 7.5×10^4 FSA cells in 100 µl HBSS were injected subcutaneously into 4 sites in the dorsal skin. In one experiment the FSA cells were injected subcutaneously in the ventral skin. Mice were monitored daily for the appearance of tumours; once the tumours were of measurable size, two bisecting diameters were measured twice a week, until the tumours reached 10 mm in diameter, or showed signs of ulceration, at which point the experiment was terminated. The total tumour area for one mouse was calculated by multiplying together the bisecting diameters for each tumour, and summing the product for each of the four tumours; the mean value for each group was then calculated.

UV sources and exposure

Three UV sources were used: a bank of two Philips TL12 lamps, which emitted a broad spectrum of UVB radiation between 280 and 360 nm with a peak at about 305 nm and produced an irradiance of 150 J m⁻² min⁻¹ at a distance of 20 cm from the source; a Philips TL01 lamp which emitted narrow-band UVB radiation between 311 and 313 nm and produced an irradiance of 120 J m⁻² min⁻¹ at a distance of 20 cm from the source; and a Dr Honle Light Tower source which emitted broad-band UVA radiation in the range 315–400 nm and produced an irradiance of 2020 J m⁻² min⁻¹ at a distance of 44 cm from the source. The output of the sources was determined using a filtered photodiode meter, which was calibrated against measurements made with a UV-visible spectroradiometer (model 742, Optronic Laboratories) across the spectral range 250–450 nm. The UVA source emitted 0.5% of its output in the UVB range. Cation X (2,7-dimethyl-diaza-(3,6)-cycloheptadiene-(1,6)-perchlorate), a gift from Dr Frank de Gruijl (University of Leiden), was used at 0.1 mg ml⁻¹ though a 1 cm light path. At this concentration the transmission of wavelengths below 340 nm was 0.0009% of the total output, and the source was considered to emit UVA-I.

Groups of 9–17 mice were irradiated twice a week for 3 weeks and FSA cells were injected subcutaneously 24 h after the final exposure; in some cases the UV exposure continued following FSA cell injection. Mice were shaved on the back at least 24 h before the first irradiation and again 24 h before the final irradiation. Control mice were shaved but not irradiated. Mice were placed in a Perspex box for the irradiation with no more than 4 mice per box to avoid shielding by littermates.

Cis-urocanic acid

Cis-UCA was prepared by irradiation of *trans*-UCA (Sigma, UK), followed by purification by thin-layer chromatography to give a greater than 99% pure preparation, as described previously.¹⁹ For topical application, *cis*-UCA was dissolved in ethanol (2 mg ml⁻¹) and 50 µl applied to shaved dorsal skin. For intradermal injection, *cis*-UCA was dissolved in sterile PBS (1 mg ml⁻¹) and 100 µl injected into shaved dorsal skin. Mice received 100 µg *cis*-UCA twice a week for 3 weeks prior to FSA injection.

A monoclonal antibody with specificity for *cis*-UCA²⁰ was diluted in PBS and 300 µl (0.5 µg protein per mouse) injected intraperitoneally 2 h prior to each irradiation with the TL12 source (1200 J m⁻²). Control mice received an isotype control antibody (IgG1; anti-Border disease virus) at the same protein concentration and times. Unirradiated controls received either *cis*-UCA antibody or control antibody.

Statistics

Statistical significance between groups was determined using the two-tailed student's *t*-test for unpaired data. A probability of less than (*p* <) 0.05 of no difference between the groups was considered significant.

Results

Following injection of the FSA cells, palpable tumours (1 mm diameter) appeared around day 4. The majority of tumours formed grew to approximately 4 mm diameter, at which point growth rate declined (around day 15). Some tumours continued to grow until they reached 10 mm diameter, at which point the mice were killed for humane reasons; mice were also killed when tumours (usually the largest) showed signs of ulceration (around day 24).

Broad-band UVB exposure enhances tumour outgrowth

A dose of 1500 J m⁻² broad-band UVB (TL12) represents 1 minimal erythral dose (MED) for C3H mice, and a single dose of 1000 J m⁻² broad-band UVB (TL12) has been shown to suppress both the CH and DTH responses in these mice.¹⁵ This dose was therefore chosen and mice were initially irradiated twice a week for 3 weeks with 1000 J m⁻² TL12. Such an exposure for 3 weeks prior to FSA cell injection had no effect on the outgrowth of the tumours compared with unirradiated controls (Fig. 1a); however, increasing the dose of UVB to 1200 J m⁻² resulted in a significant increase in the size of the tumours. Continuing to irradiate the mice with 1200 J m⁻² after injection of FSA did not further increase the UV-enhanced outgrowth of the tumours. The experiment was repeated with similar results.

Fig. 1b demonstrates that the effect of UVB on tumour outgrowth was a local one. Exposure of mice to 1500 J m⁻² over a period of 3 weeks, a dose that increased the outgrowth of FSA cells injected into UV-irradiated dorsal skin, had no effect on the outgrowth of FSA cells implanted into unirradiated ventral skin.

UVA-I and narrow-band TL01 exposure do not enhance tumour outgrowth

In a previous study, it was demonstrated that a single dose of 1000 J m⁻² UVA-I suppressed the DTH response in C3H mice, while a dose of 500,000 J m⁻² (1 MED) was required for suppression of CH.¹⁵ The same study demonstrated that a single dose of 10,000 J m⁻² TL01 (1 MED) suppressed the DTH response in these mice. Doses exceeding those necessary to suppress the hypersensitivity responses were therefore chosen. Fig. 2 shows that exposure of mice twice a week for 3 weeks to 10,000 or 500,000 J m⁻² UVA-I radiation prior to FSA cell

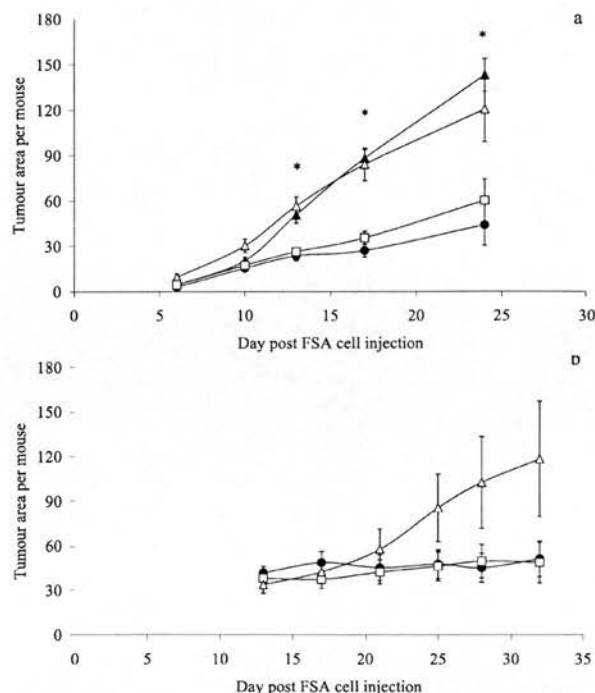


Fig. 1 Growth of FSA cells in C3H mice following broadband UVB (TL12) exposure. (a) Groups of 17 mice were irradiated twice a week for 3 weeks with 0 (●), 1000 (□) or 1200 (Δ) J m⁻² TL12. FSA cells were injected 24 h after the final UV exposure. One group continued to receive 1200 J m⁻² TL12 twice a week following FSA cell injection (▲). * shows a significant difference ($p < 0.05$) between the unirradiated group and the groups irradiated with 1200 J m⁻². (b) Groups of 12 mice were irradiated twice a week for 3 weeks with 1500 J m⁻² TL12. The ventral skin was shaved 24 h before the final UV exposure; FSA cells were injected in 4 ventral sites (□) or 4 dorsal sites (Δ) 24 h after the final UV exposure. Control mice were not irradiated (●).

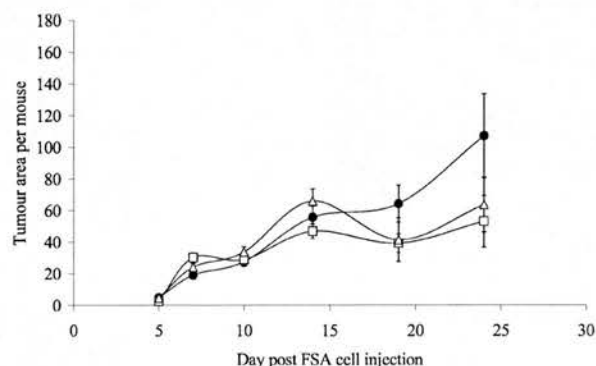


Fig. 2 Growth of FSA cells in C3H mice following UVA-I exposure. Groups of 9 mice were irradiated twice a week for 3 weeks with 0 (●), 10,000 (□) or 500,000 (Δ) J m⁻² UVA-I. FSA cells were injected 24 h after the final UV exposure.

injection had no effect on the size of the resulting tumours, compared with unirradiated controls. Irradiation of mice for 3 weeks with 10,000 (data not shown) or 14,000 J m⁻² TL01 had no effect on the outgrowth of subsequently injected FSA cells (Fig. 3). Continuing the irradiation protocol following tumour cell injection however, resulted in a significant increase in the size of the resulting tumours, beginning on day 18 post-injection.

Cis-UCA does not enhance tumour outgrowth

Reeve *et al.*²¹ demonstrated that topical application of UCA during UV-induced carcinogenesis increased both the number

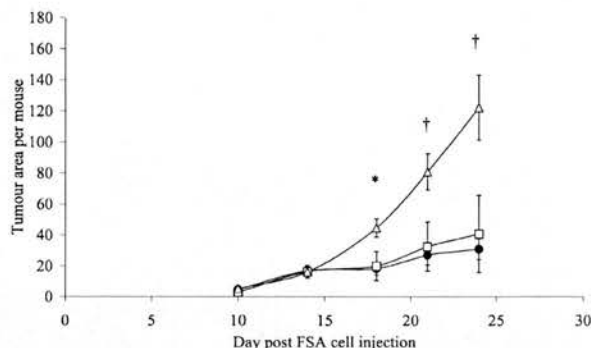


Fig. 3 Growth of FSA cells in C3H mice following narrow-band UVB (TL01) exposure. Groups of 12 mice were irradiated twice a week for 3 weeks with 0 (●) or 14,000 (□) J m⁻² TL01. FSA cells were injected 24 h after the final UV exposure. One group continued to receive 14,000 J m⁻² TL01 twice a week following FSA cell injection (Δ). * and † show a significant difference ($p < 0.05$ and $p < 0.005$ respectively) between the unirradiated group and the group irradiated with TL01 before and after FSA cell injection.

and state of malignancy of the resulting tumours. Topical application of *cis*-UCA also suppressed the DTH response to herpes simplex virus in C3H mice,¹⁸ while injection of a monoclonal antibody to *cis*-UCA prior to UV-irradiation reversed the UV-induced suppression of DTH to alloantigen.²² We therefore investigated whether *cis*-UCA was a mediator in UV-enhanced tumour outgrowth.

Topical application of 100 μg *cis*-UCA onto shaved dorsal skin for 3 weeks prior to FSA cell injection had no effect on the outgrowth of FSA cells compared with mice that received the vehicle only (Fig. 4a). This experiment was repeated with similar results. To exclude the possibility that this was due to a lack of uptake of *cis*-UCA through the skin, *cis*-UCA was injected intradermally twice a week for 3 weeks; there was no significant difference in tumour size between these mice and controls (Fig. 4a).

Injection of 0.5 μg *cis*-UCA antibody prior to each exposure with 1200 J m⁻² TL12 had no effect on the increase in tumour size seen following UV exposure (Fig. 4b); increasing the dose of *cis*-UCA antibody to 5 μg protein per mouse gave a similar result (data not shown). Injection of *cis*-UCA antibody also had no effect on the outgrowth of tumours in unirradiated mice (Fig. 4b).

Discussion

Many UV-induced tumours in mice are rejected upon transplantation into normal syngeneic hosts; however, these tumours can grow progressively if transplanted into hosts whose immune systems have been suppressed by UVR or another method. These immunogenic properties are not restricted to murine UV-induced skin tumours; an increase in skin cancers on the sun-exposed skin of patients taking immunosuppressive drugs has been observed,^{23,24} suggesting that many potential cancer cells are destroyed by individuals with an intact immune system. Although the nature of the immune response to skin cancers is not well understood (for review see ref. 25), there is evidence that UV-enhanced outgrowth of implanted skin tumours is accompanied by induction of suppressor T cells in the spleen and lymph nodes.²⁶

In the current study, irradiation of mice twice a week for 3 weeks with 1200 J m⁻² broad-band UVB (280–360 nm) resulted in enhanced outgrowth of FSA cells injected into the UV irradiated dorsal skin. There may be a minimal dose requirement as 1000 J m⁻² had no effect. The enhanced outgrowth was not seen when FSA cells were injected into the unirradiated ventral skin of irradiated mice; the effect of the broad-band UVB was therefore a local one. This result is in

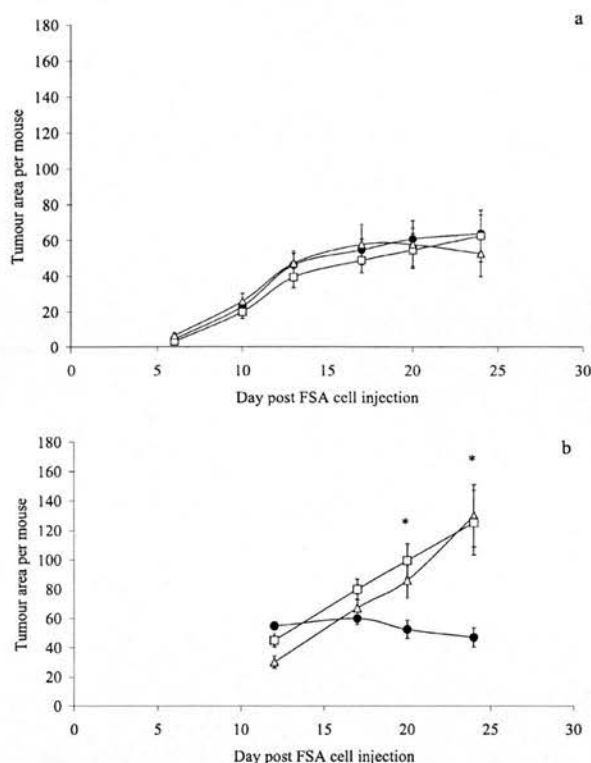


Fig. 4 Effect of *cis*-UCA on growth of FSA cells in C3H mice. (a) Groups of 10 mice were treated topically twice a week for 3 weeks with *cis*-UCA (□) or vehicle only (●); alternatively mice were injected subcutaneously with *cis*-UCA (Δ). FSA cells were injected 24 h after the final treatment. (b) Groups of 14 mice were irradiated twice a week for 3 weeks with 1200 J m⁻² broad-band UVB (TL12); two hours before each irradiation mice were injected i.p. with *cis*-UCA antibody (Δ) or an isotype control antibody (□). Control mice received *cis*-UCA antibody (●) but were not irradiated. * shows a significant difference (p < 0.05) between the unirradiated group and the irradiated groups.

agreement with the results of Romerdahl *et al.*,²⁷ where unshaven mice were irradiated with one ear covered; subsequently injected melanoma cells showed increased outgrowth in the UV-exposed ear but not in the ear that had been covered. Previous studies have indicated that the effect of UVR is systemic, as the suppression of immune responses to implanted⁴ or primary UV-induced²⁸ tumours can be transferred from irradiated to unirradiated mice by spleen and lymphoid cells. In an experiment by Fisher and Kripke,²⁶ where 2 mice, one UV-irradiated and the other unirradiated, were joined parabiotically, implanted tumours grew progressively in both mice. In that study, the mice were irradiated for at least 3 months prior to the parabiosis, and each dose was 10,000 J m⁻² (FS40 Sunlamps); the lower dose of UV used in the present study might not have been sufficient to cause systemic suppression of anti-tumour responses.

In previous reports investigating the effects of UVA radiation on immune responses, a variety of sources and filters have been used, making it difficult to compare one study with another. We chose to exclude wavelengths below 340 nm by a Cation X screen. It was shown that prior exposure to the UVA-I lamp had no effect on the size of the FSA tumours. This supports the findings by Aubin *et al.*,²⁹ that prior exposure of mice to UVA does not result in progressive growth of subsequently implanted melanomas. In a previous study⁴ exposure of BALB/c mice to FS40 Sunlamps, with 87% of their output between 255–340 nm with a main peak at 313 nm, increased the growth of subsequently implanted UV-induced fibrosarcomas. Use of filters that removed 99% of wavelengths below 275 nm, or 99% of wavelengths below 315 nm, demonstrated that the most

effective wavelengths for UV-enhanced tumour outgrowth most probably lay within the UVB range. A study by de Gruijl *et al.*⁵ demonstrated that for induction of skin cancer in hairless mice, the most effective wavelength was 293 nm, which lies within the UVB range; however, wavelengths within the UVA range were also effective, although to a much lesser extent. In a model using the opossum *Monodelphis domestica*, exposure to UVA (over 320 nm) 3 times a week for 87 weeks gave rise to non-melanoma skin tumours in the UV-irradiated sites in 50% of the animals.³⁰

It is possible that the dose of UVA used was not high enough to cause suppression of anti-tumour responses; alternatively it may be that, although UVA can induce tumours, these wavelengths do not initiate the suppressive events leading to increased tumour cell outgrowth. The UV-induced tumours that are reported as highly antigenic were all induced by exposure to wavelengths in the UVB range;¹ it is not known whether UVA-induced tumours demonstrate the same degree of antigenicity.

The TL01 lamp has an output mainly within the UVB range, with a peak (51%) at 311 nm. Exposure of mice to 14,000 J m⁻² TL01 for 3 weeks prior to inoculation with FSA cells had no effect on the outgrowth of the subsequent tumours; however, in mice that continued to be irradiated after tumour cell injection, the resulting tumours were significantly increased in size compared with those in unirradiated mice. It is not clear whether this effect of the TL01 exposure was on the FSA cells themselves, or on the host. A single dose of 10,000 J m⁻² TL01 (1 MED) is sufficient to cause suppression of the DTH response in these mice; however a dose of 50,000 J m⁻² is necessary to suppress the CH response.¹⁵ Although the mice received a total of 84,000 J m⁻² TL01 prior to FSA cell injection, it may be that higher or continuing doses are required to induce increased outgrowth of inoculated tumour cells.

In this study, *cis*-UCA was not found to have a major role in UV-induced suppression of immune responses to FSA cells. Two previous studies have suggested that *cis*-UCA may be important in the pathogenesis of skin cancer. Topical application of UCA prior to irradiation with solar-simulating UV during UV carcinogenesis increased both the number and malignancy of the resultant tumours, compared with mice receiving only UV.²⁰ More recently, Beissert *et al.*³¹ used tumour antigen-pulsed epidermal cells to immunise mice against a S1905 spindle cell tumour. Mice that had received epidermal cells, incubated in *cis*-UCA prior to antigen pulsing, were unable to reject subsequently injected tumour cells; these mice also showed a significantly suppressed DTH response to the tumour cells. This inhibition by *cis*-UCA of tumour antigen presentation by epidermal cells was completely reversed by addition of IL-12 before, during or after incubation of the cells with *cis*-UCA.³²

The apparently conflicting results of these studies with the present one could be due to the very different protocols and endpoints. Our results showed that an antibody to *cis*-UCA did not counteract the UV-enhanced tumour growth, nor did treatment of mice with *cis*-UCA increase the growth of subsequently injected FSA cells. The increase in carcinogenesis seen by Reeve *et al.*²⁰ may be caused by an increase in induction of tumours by UCA, rather than promotion by suppression of anti-tumour immune responses. As the UCA was applied prior to each irradiation, it is possible that products were formed from the photo-oxidation of UCA, which is induced by UVB, but not UVA, radiation; these photo-oxidation products are thought to have a role in UV-induced immune suppression.³³

The amount of *cis*-UCA present in the skin following the 3 week irradiation protocol was not analysed in the present study. Previous measurements had shown that, after exposure to a single dose of 1000 J m⁻² TL12, or 10,000 J m⁻² TL01, the irradiated skin contained approximately 42% *cis*-UCA, compared to 1.7% in unirradiated skin.¹⁵ A single dose of 500,000 J

m⁻² UVA-I resulted in only 17% formation of *cis*-UCA. During chronic irradiation of mice with doses of 1000 J m⁻² TL12, or 3000 J m⁻² TL01 three times each week, an increase in *cis*-UCA was seen after the first week, and a maximum of 35% and 38% *cis*-UCA was reached with the TL01 and TL12 lamps after 1 and 2 weeks respectively.³⁴ When these percentages were compared with suppression of hypersensitivity responses, no correlation could be established.

Moodycliffe *et al.*²² reported that injection of a monoclonal antibody specific for *cis*-UCA prior to UV-irradiation of mice completely reversed the UV-induced suppression of the DTH response to allogeneic spleen cells, although it had no effect on UVB-induced suppression of the CH response to oxazalone. In another report, the antibody to *cis*-UCA reversed the DTH response to herpes simplex virus, but again had no effect on the CH response to oxazalone.³⁵ Since UV-induced suppression appears to have different pathways for different antigens, the present study shows that UCA is not an important photoreceptor in the cascade of immune response reduction during outgrowth of tumour cells.

In conclusion, we have shown that irradiation of mice with the broad-band UVB lamp was the most effective in increasing the outgrowth of injected FSA cells; this effect was a local one, as FSA cells injected into an unirradiated site of irradiated mice did not show increased growth. Despite the carcinogenic and immunosuppressive properties of UVA, irradiation of mice with UVA-I did not cause increased growth of subsequently injected tumour cells. Although *cis*-UCA may be important in the early stages of UV-induced carcinogenesis, it was not a critical mediator of UV-induced suppression of immune responses to injected tumour cells.

Acknowledgements

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References

- 1 M. L. Kripke, Antigenicity of murine skin tumours induced by ultraviolet light, *J. Natl. Cancer Inst.*, 1974, **53**, 1333–1336.
- 2 M. L. Kripke and M. S. Fisher, Immunologic parameters of ultraviolet carcinogenesis, *J. Natl. Cancer Inst.*, 1976, **57**, 211–215.
- 3 C. W. Spellman and R. A. Daynes, Modification of immunological potential by ultraviolet radiation: II. Generation of suppressor cells in short-term UV-irradiated mice, *Transplantation*, 1977, **24**, 120–126.
- 4 E. C. De Fabo and M. L. Kripke, Wavelength dependence and dose-rate independence of UV radiation-induced immunologic unresponsiveness of mice to a UV-induced fibrosarcoma, *Photochem. Photobiol.*, 1980, **32**, 183–188.
- 5 F. R. de Grujil, H. J. C. M. Sterenborg, P. D. Forbes, R. E. Davies, C. Cole, G. Kelfkens, H. vanweelden, H. Slaper and J. C. van der Leun, Wavelength dependence of skin-cancer induction by ultraviolet-irradiation of albino hairless mice, *Cancer Res.*, 1993, **53**, 53–60.
- 6 S. E. Ullrich, The role of epidermal cytokines in the generation of cutaneous immune-reactions and ultraviolet radiation-induced immune suppression, *Photochem. Photobiol.*, 1995, **62**, 389–401.
- 7 A. A. El-Ghorr, F. Pierik and M. Norval, Comparative potency of different UV sources in reducing the density and antigen-presenting capacity of Langerhans cells in mice, *Photochem. Photobiol.*, 1994, **60**, 256–261.
- 8 W. Goettsch, H. M. H. Hurks, J. Garssen, A. M. Mommaas, W. Slob, J. Hoekman, F. Pierik, P. J. M. Roholl and H. Van Loveren, Comparative immunotoxicology of ultraviolet B exposure – I. Effects of *in vitro* and *in situ* ultraviolet B exposure on the functional activity and morphology of Langerhans cells in the skin of different species, *Br. J. Dermatol.*, 1998, **139**, 230–238.
- 9 A. M. Moodycliffe, D. Nghiem, G. Clydesdale and S. E. Ullrich, Immune suppression and skin cancer development: regulation by NKT cells, *Nat. Immunol.*, 2000, **1**, 521–525.
- 10 A. Schwarz, S. Beissert, K. Grosse-Heitmeyer, M. Gunzer, J. A. Bluestone, S. Grabbe and T. Schwarz, Evidence for functional

relevance of CTLA-4 in ultraviolet-radiation-induced tolerance, *J. Immunol.*, 2000, **165**, 1824–1831.

- 11 M. L. Kripke, P. A. Cox, L. G. Alas and D. B. Yarosh, Pyrimidine dimers in DNA initiate systemic immunosuppression in UV-irradiated mice, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 7516–7520.
- 12 E. C. De Fabo and F. P. Noonan, Mechanism of immune suppression by ultraviolet irradiation *in vivo* 1. Evidence for the existence of a unique photoreceptor in skin and its role in photoimmunology, *J. Exp. Med.*, 1983, **157**, 84–98.
- 13 T. Mohammad, H. Morrison and H. HogenEsch, Urocanic acid photochemistry and photobiology, *Photochem. Photobiol.*, 1999, **69**, 115–135.
- 14 V. E. Reeve, M. Bosnic, C. Boehm-Wilcox, N. Nishimura and R. D. Ley, Ultraviolet A radiation (320–400 nm) protects hairless mice from immunosuppression induced by ultraviolet B radiation (280–320 nm) or *cis*-urocanic acid, *Int. Arch. Allergy Immunol.*, 1998, **115**, 316–322.
- 15 A. A. El-Ghorr and M. Norval, The UV waveband dependencies in mice differ for the suppression of contact hypersensitivity, delayed-type hypersensitivity and *cis*-urocanic acid formation, *J. Invest. Dermatol.*, 1999, **112**, 757–762.
- 16 I. Iwai, M. Hatao, M. Naganuma, Y. Kumano and M. Ichihashi, UVA-induced immune suppression through an oxidative pathway, *J. Invest. Dermatol.*, 1999, **112**, 19–24.
- 17 H. D. Suit and A. Kastelan, Immunologic status of host and response of a methylcholanthrene-induced sarcoma to local x-irradiation, *Cancer*, 1970, **26**, 232–238.
- 18 W. H. McBride, J. D. Thacker, S. Comora, J. S. Economou, D. Kelley, D. Hogge, S. M. Dubinett and G. J. Dougherty, Genetic modification of a murine fibrosarcoma to produce interleukin 7 stimulates host cell infiltration and tumour immunity, *Cancer Res.*, 1992, **52**, 3931–3937.
- 19 M. Norval, T. J. Simpson, E. Bardshiri and S. E. M. Howie, Urocanic acid analogs and the suppression of the delayed-type hypersensitivity response to herpes-simplex virus, *Photochem. Photobiol.*, 1989, **49**, 633–639.
- 20 A. M. Moodycliffe, M. Norval, I. Kimber and T. J. Simpson, Characterization of a monoclonal antibody to *cis*-urocanic acid – detection of *cis*-urocanic acid in the serum of irradiated mice by immunoassay, *Immunology*, 1993, **79**, 667–672.
- 21 V. E. Reeve, G. E. Greenoak, P. J. Canfield, C. Boehm-wilcox and C. H. Gallagher, Topical urocanic acid enhances UV-induced tumor yield and malignancy in the hairless mouse, *Photochem. Photobiol.*, 1989, **49**, 459–464.
- 22 A. M. Moodycliffe, C. D. Bucana, M. L. Kripke, M. Norval and S. E. Ullrich, Differential effects of a monoclonal antibody to *cis*-urocanic acid on the suppression of delayed and contact hypersensitivity following ultraviolet irradiation, *J. Immunol.*, 1996, **157**, 2891–2899.
- 23 A. L. P. Caforio, A. B. Fortina, A. Gambino, S. Piaserico, G. Feltrin, F. Tona, E. Pompei, L. Testolin, F. Gai, A. Angelini, D. Casarotto and A. Peserico, High rejection score in the first year and risk of skin cancer in heart transplantation, *Transplant. Proc.*, 2001, **33**, 1608–1609.
- 24 J. Boyle, R. M. Mackie, J. D. Briggs, B. J. R. Junor and T. C. Aitchison, Cancer, warts and sunshine in renal-transplant patients – a case control study, *Lancet*, 1984, **1**, 702–705.
- 25 F. R. de Grujil, UV radiation and tumour immunity, *Methods*, 2002, **28**, 122–129.
- 26 M. S. Fisher and M. L. Kripke, Further studies on the tumour-specific suppressor cells induced by ultraviolet radiation, *J. Immunol.*, 1978, **121**, 1139–1144.
- 27 C. A. Romerdahl, C. Donawho, I. J. Fidler and M. L. Kripke, Effect of ultraviolet-B radiation on the *in vivo* growth of murine melanoma cells, *Cancer Res.*, 1988, **48**, 4007–4010.
- 28 M. S. Fisher and M. L. Kripke, Suppressor T lymphocytes control the development of primary skin cancers in ultraviolet-irradiated mice, *Science*, 1982, **216**, 1133–1134.
- 29 F. Aubin, C. K. Donawho and M. L. Kripke, Effect of psoralen plus ultraviolet A radiation on *in vivo* growth of melanoma cells, *Cancer Res.*, 1991, **51**, 5893–5897.
- 30 R. D. Ley, Dose response for ultraviolet radiation A-induced focal melanocytic hyperplasia and nonmelanoma skin tumors in *Monodelphis domestica*, *Photochem. Photobiol.*, 2001, **73**, 20–23.
- 31 S. Beissert, T. Mohammad, H. Torri, A. Lonati, Z. M. Yan, H. Morrison and R. D. Granstein, Regulation of tumor antigen presentation by urocanic acid, *J. Immunol.*, 1997, **159**, 92–96.
- 32 S. Beissert, D. Ruhlemann, T. Mohammad, S. Grabbe, A. El-Ghorr, M. Norval, H. Morrison, R. D. Granstein and T. Schwarz, IL-12 prevents the inhibitory effects of *cis*-urocanic acid on tumor antigen presentation by Langerhans cells: Implications for photocarcinogenesis, *J. Immunol.*, 2001, **167**, 6232–6238.

- 33 A. Kammeyer, T. A. Eggelte, H. Overmars, A. Bootsma, J. D. Bos and M. B. M. Teunissen, Oxidative breakdown and conversion of urocanic acid isomers by hydroxyl radical generating systems, *Biochim. Biophys. Acta-Gen. Subjects*, 2001, **1526**, 277–285.
- 34 A. A. El-Ghorr, M. Norval, M. B. Lappin and J. C. Crosby, The effect of chronic low-dose UVB radiation on Langerhans cells, sunburn cells, urocanic acid isomers, contact hypersensitivity and serum immunoglobulins in mice, *Photochem. Photobiol.*, 1995, **62**, 326–332.
- 35 A. A. El-Ghorr and M. Norval, A monoclonal antibody to *cis*-urocanic acid prevents the ultraviolet-induced changes in Langerhans cells and delayed-hypersensitivity responses in mice, although not preventing dendritic cell accumulation in lymph-nodes draining the site of irradiation and contact hypersensitivity responses, *J. Invest. Dermatol.*, 1995, **105**, 264–268.

Exposure to multiple doses of UVB radiation reduces the numbers of epidermal Langerhans cells and lymph node dendritic cells in mice

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Immune suppression following UVB irradiation is partly attributed to the effects of the exposure on antigen-presenting cells. Following a single UVB irradiation, there is a decrease in epidermal Langerhans cell numbers; this is accompanied by an increase in the number of dendritic cells (DC) in lymph nodes draining the irradiated site. We investigated whether a similar effect occurred following multiple UVB exposures. Mice were irradiated on their ears and shaved dorsal skin twice a week for 3 weeks. After the final exposure, the number of ATPase⁺ Langerhans cells in epidermal sheets prepared from the ears was found to be decreased by 33% compared to unirradiated controls. The number of DC in the draining lymph nodes (DLN) did not increase as might have been expected; rather, a significant decrease of approximately 30% in DC numbers in the DLN of UVB-irradiated mice compared with unirradiated controls occurred. This decrease in antigen-presenting cells in both the epidermis and the DLN may be an important contributing factor to the immune suppression that follows multiple UVB exposures.

Introduction

Exposure to ultraviolet radiation (UVR) causes suppression of T-cell responses, such as the contact hypersensitivity (CH) response, in both mice and humans.^{1,2} During contact sensitisation, hapten-bearing Langerhans cells (LC), normally resident in the epidermis, migrate to the draining lymph nodes (DLN) to induce an immune response.³ There is evidence that UVR-induced suppression of immune responses may be due, at least partly, to effects on antigen-presenting cells of the skin. LC are both decreased in number and altered morphologically following UV irradiation.⁴ UV exposure of human skin results in increased numbers of LC in suction blister fluid, indicating that UV-induced migration takes place.⁵ In another study, UV irradiation [1 minimal erythema dose (MED)] of the skin of human volunteers increased the lymph flow and cell output in an afferent lymph vessel.⁶ Moodycliffe *et al.*⁷ demonstrated that the number of dendritic cells (DC) in the skin DLN of unsensitised mice increased as a result of irradiation with 1 MED UVB, with a peak at 42 h after the exposure. Taken together, these studies suggest that, following a single dose of UV, some LC migrate from the epidermis through the afferent lymph and accumulate in the DLN. How this change relates to the immune suppression that follows UV exposure is unclear. One consequence could be a decrease in the numbers of LC in the skin available to take up antigen. Alternatively, some LC migrating from UV-irradiated skin show DNA damage⁸ which might lead to altered antigen presentation by these cells. *In vitro* UV exposure of LC affects the expression of co-stimulatory molecules such as CD86 (B7.2) and intercellular adhesion molecule-1;^{8,9} a decrease in these molecules could result in a tolerogenic, rather than an immunogenic, signal being generated. Finally, it has been demonstrated that following UV irradiation, there is an influx of inflammatory cells, including macrophages, into the skin;¹⁰ presentation of antigen by these cells gives rise to a suppressive, rather than an active, immune response.¹¹

Although the effects of a single, frequently erythemogenic, UV exposure on immune responses are useful for examining the

complex processes involved in UV-induced immune suppression, multiple exposures over a period of weeks are perhaps more relevant to the sunlight exposure naturally received by humans. Such a chronic protocol can also remove the need to use high doses of UV. Whereas a single dose of 1250 J m⁻² was not sufficient to reduce epidermal LC numbers in C3H mice,¹² the numbers were decreased following exposure 3 times weekly to a dose of 500 J m⁻² for 1–3 weeks.¹³ In the latter study, tanning of the UV exposed mice was seen after 3 weeks exposure, and it is not known at present whether such an adaptive response could affect the induction of suppression.

The purpose of the present study was to assess whether a 3 week UV protocol had similar effects to an acute UV exposure on the migration of LC in unsensitised mice. Following exposure of C3H mice to UVB for 3 weeks, the numbers of both epidermal LC and DC in LN draining the skin were counted. The expression of co-stimulatory molecules on the DC was also assessed. The *in vivo* CH response and the ability of LN cells to undergo spontaneous proliferation *in vitro* were tested as measures of immune function.

Materials and methods

Mice

Female C3H/HeN (H-2k) mice aged 6–10 weeks were obtained from the Medical Faculty Animal Area, University of Edinburgh; they were housed in a room where ambient light was regulated on a 12 h light/dark cycle and had free access to food and water. Room lights were shielded such that any contaminating UV wavelengths were filtered out. All experiments were performed according to the ethical guidelines of the University of Edinburgh and the Home Office.

UV exposure

Mice were irradiated under a bank of two Philips TL12 lamps, which emit a broad spectrum of UVB radiation between 280 and 360 nm, with a peak at about 305 nm, and produced an

irradiance of $150 \text{ J min}^{-1} \text{ m}^{-2}$ at a distance of 20 cm from the source. The output of the lamps was determined using a filtered photodiode meter, which was calibrated against measurements made with a UV-visible spectroradiometer (Optronic Laboratories model 724) across the spectral range 250–450 nm. The backs of the mice were shaved at least 24 h before the first irradiation, and the mice irradiated with 1500 J m^{-2} twice a week (Tuesday and Friday) for 3 weeks. Mice were placed in a Perspex box for the irradiation, with no more than 4 mice per box to avoid shielding by littermates. Control mice were shaved but not irradiated.

Epidermal Langerhans cells

UVB-irradiated and unirradiated control mice were killed by cervical dislocation 24 h after the final irradiation, and their ears removed and split. The dorsal side was floated in 0.76% tetrasodium ethylenediamine tetraacetic acid for 2 h at 37°C , and the epidermal sheets removed. The number of LC in the epidermal sheets was determined by staining for adenine triphosphatase (ATPase), using adenine diphosphate (ADP) as a substrate.¹⁴ Following staining, the sheets were rinsed in tap water and mounted onto a glass slide under a cover slip in 50% glycerol. The number of ATPase⁺ cells in 10 fields per epidermal sheet was counted (1 field = 0.1 mm^2), with 4 sheets per group.

Lymphoproliferation of draining lymph node cells and dendritic cell enrichment

Mice were killed by cervical dislocation 42 h after the final UV irradiation or were unirradiated (8 mice per group). Their auricular, axillary and inguinal LN were collected and pooled in RPMI medium (Gibco BRL, Paisley, UK) containing 10% foetal bovine serum (Gibco BRL) and 10 mM HEPES buffer (Sigma, Poole, UK). Single cell suspensions were prepared by mechanical disaggregation through a nylon cell strainer (Fred Baker Scientific, Runcorn, UK) and washed once. Viable cells were counted by Trypan Blue exclusion. For the lymphoproliferation assay, cells were resuspended to $2 \times 10^6 \text{ cells ml}^{-1}$ and 200 μl of this seeded into wells of a 96-well round-bottom culture plate (Iwaki, Asahi Techno Glass, Tokyo, Japan) (5 replicates per group). Cells were radioactively pulsed by adding 0.7 μCi ^3H -methyl thymidine (Amersham Life Science, Amersham, UK) per well and incubated for 24 h at 37°C in a humid atmosphere of 5% CO_2 in air. Cells were harvested onto filter mats and ^3H -thymidine incorporation measured in counts per minute using a scintillation counter (Canberra Packard, Zurich, Switzerland). The mean count of the 5 replicates for each group was calculated.

The LN cell suspensions were enriched for DC using the method of Macatonia *et al.*¹⁵ Briefly, 4×10^7 LN cells in 8 ml medium were underlaid with 2 ml metrizamide (14.5%, Sigma, UK). Following 15 min centrifugation at 600g, the interface layer was collected, washed and resuspended in a minimal amount of medium. The number of DC was assessed by morphological examination by light microscopy; at least 5 counts for each group were made and the mean number of DC per LN was calculated.

Phenotyping of DC by flow cytometry

DC-enriched LN cell suspensions were prepared as above and 4×10^5 cells incubated on ice for 45 min with an isotype control antibody (rat anti-human CD8, Serotec, Oxford, UK) or monoclonal antibodies recognising Ia or CD86 (rat anti-mouse, Serotec). The cells were washed once with 1 ml RPMI-HEPES, resuspended in RPMI-HEPES and incubated with an affinity-purified F(ab')₂ goat anti-rat IgG-FITC conjugate (100 μl of 1 : 100 dilution; Serotec) for 45 min on ice. Cells were washed again, resuspended in 1 ml phosphate-buffered saline and analysed using a Coulter XL flow cytometer. Cells were identified

first using forward scatter and side angle light scatter to quantify their size and granularity. Gates were placed around the entire cell population and around cells with low forward and side angle scatter (lymphocytes) and around the larger cells. The events within each region were displayed on histograms of log fluorescence intensity (x-axis) against cell count (y-axis). Isotype controls were routinely set at 1% and a minimum of 20 000 events was accumulated in the region around the entire cell population.

Annexin V binding of DC-enriched LN cell populations was determined by incubation of the cells for 3 min at room temperature with FITC-conjugated annexin V (Boehringer, Ingelheim, Germany) diluted 1 : 500 in Hanks' balanced salt solution containing 5 μM CaCl_2 . Cells were analysed immediately by flow cytometry, as described above.

Measurement of CH response

The CH response to oxazalone was measured in irradiated and unirradiated mice by a standard technique, as outlined previously.¹⁶ Six mice per group received a sensitising dose of 50 μl 1% oxazalone in an acetone-olive oil (4 : 1) vehicle on their shaved backs, 3 days after the final UV exposure. An unirradiated negative control group received 50 μl of the vehicle alone. Eight days after the sensitisation step, ear thicknesses were measured and all the mice were challenged with 25 μl per ear of 0.25% oxazalone on the dorsal surface. Ear swelling was measured 24 h later and the mean ear increase for each mouse was calculated, followed by the mean increase for each group of mice.

Statistics

Statistical significance between groups was determined using the two-tailed student's t-test for unpaired data. A probability of less than ($p <$) 0.05 of no difference between the groups was considered significant.

Results

Exposure to UVB for 3 weeks reduces epidermal LC numbers

It has been reported that a single exposure to 5000 J m^{-2} UVB (TL-12) reduces the number of LC in the epidermis by around 28%.¹² This depletion was demonstrated to be dose dependent, with a dose of 1250 J m^{-2} being insufficient to cause a significant drop in LC numbers. To determine the effect of multiple UV doses over a period of 3 weeks on epidermal LC numbers, mice were irradiated twice a week for 3 weeks with 1500 J m^{-2} broad-band UVB. The ears were removed 24 h after the final irradiation and the frequency of LC in the epidermis determined by counting the number of ATPase⁺ cells with dendritic morphology. Fig. 1 shows that the number of epidermal LC decreased by approximately 33%, a significant difference compared to the unirradiated control mice.

Exposure to UVB for 3 weeks decreases the number of DC in the DLN

Moodycliffe *et al.*⁷ demonstrated that a single exposure of the ears of C3H mice to 1440 J m^{-2} broad-band UVB induced an increase in the number of DC in the auricular LN, which was maximal at around 42 h following the irradiation. In the present study, mice were shaved and exposed to a single dose of 1500 J m^{-2} UVB. This resulted in an increase in the number of DC in the LN draining the irradiated dorsal surface from 4575 to 6595 per LN. To determine whether the same effect was seen in mice following multiple UV exposures, mice were exposed to 1500 J m^{-2} UVB twice a week for 3 weeks and LN draining both the ears and back were pooled and enriched for DC. The results of 4 separate experiments are shown in Table 1. The number of

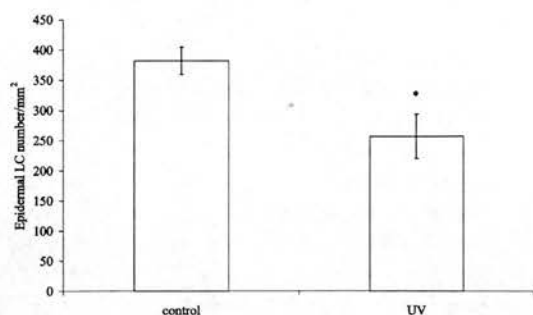


Fig. 1 Effect of 3 weeks UVB exposure on epidermal LC numbers. Mice (2 per group) were irradiated twice a week for 3 weeks and their ears removed 24 h after the final UV exposure. Control mice were not irradiated. The epidermal sheets were removed, fixed and stained using ADP-lead; the number of ATPase⁺ DC was counted in 40 fields of 1 mm² each. Results are expressed as the mean of 40 fields, error bars show the SEM. The asterisk indicates a significant ($p < 0.05$) difference between the irradiated and unirradiated groups.

Table 1 Effect of UVB irradiation on DC numbers in LN draining the ears and shaved backs of mice. Mice (8 per group) were irradiated twice a week for 3 weeks and their LN removed 42 h after the final UV exposure. Control mice were not irradiated. Numbers in parentheses are the SEM

Experiment	DC count per lymph node		
	Control	UV-irradiated	Decrease (%)
1	11 101	9009	19
2	7888	4776	39
3	12 295	7096	42
4	10 425	7997	16
Mean	10 427 (930)	7219 (903) ^a	29

^a Shows a significant difference ($p < 0.05$) between the control and the irradiated groups.

DC in the LN from irradiated mice was consistently less than the number in the LN from control mice. The mean for each group was calculated and a t-test showed that exposure of mice to UV for 3 weeks significantly ($p < 0.05$) decreased the number of DC in the DLN.

To assess whether the decrease in DC numbers in the LN of irradiated mice was due to increased cell death, the level of annexin V binding by the DC-enriched cell populations was measured. DC from control and irradiated mice demonstrated similar levels of annexin V binding (Fig. 2), with an average (from two experiments) of 72% positive cells for control DC and 74% for DC from irradiated mice.

Exposure to UVB for 3 weeks has no effect on DC surface molecules

Lappin *et al.*¹⁷ demonstrated that, in mice that were contact sensitised, prior exposure to a single dose of UVB had no effect on the expression of several cell surface molecules on DC enriched from DLN, compared with unirradiated mice. We assessed whether chronic UV exposure affected the expression of cell surface markers involved in antigen presentation in unsensitised mice. DLN cells from unirradiated mice and from mice that had been irradiated over a 3 week period were enriched for DC, and the expression of Ia and CD86 determined by flow cytometry. No difference was seen in the expression of either Ia [Fig. 3(a)] or CD86 [Fig. 3(b)] between mice exposed to multiple doses of UV and unirradiated controls. This experiment was repeated twice with similar results.

Exposure to UVB for 3 weeks has no effect on the spontaneous proliferation of DLN cells

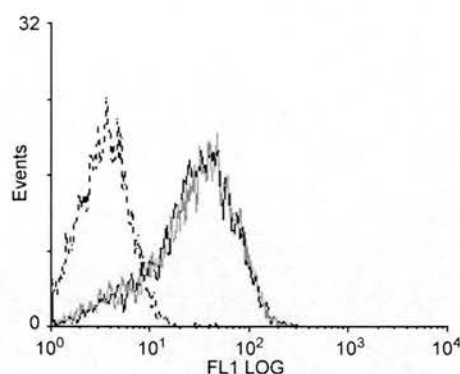


Fig. 2 Effect of 3 weeks UVB exposure on annexin V binding to DC. Mice (8 per group) were irradiated twice a week for 3 weeks and their LN removed 42 h after the final exposure. Control mice were not irradiated. Following DC enrichment of DLN cells, the enriched populations were stained with FITC-conjugated annexin V. The fluorescence intensity of the gated DC population is shown for control (continuous black line) and irradiated mice (grey line). The fluorescence intensity of DC from unirradiated mice stained with a control antibody is also shown (dotted black line).

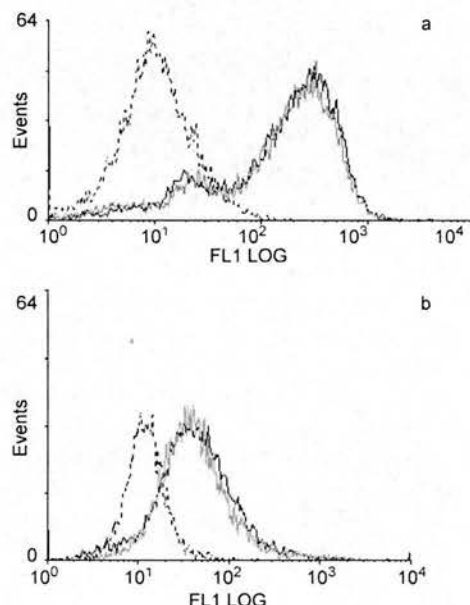


Fig. 3 Effect of 3 weeks UVB exposure on expression of cell surface markers on DC. Mice (8 per group) were irradiated twice a week for 3 weeks and their LN removed 42 h after the final exposure. Control mice were not irradiated. Following DC enrichment of DLN cells, the enriched populations were stained for the presence of Ia (a) and CD86 (b), and analysed by flow cytometry. The fluorescence intensity of the gated DC population is shown for control (continuous black line) and irradiated mice (grey line). The fluorescence intensity of DC from unirradiated mice stained with a control antibody is also shown (dotted black line).

LN cells that are removed from normal mice have a low spontaneous proliferation *in vitro*; application of a contact sensitizer to the skin results in increased proliferation of cells from LN draining the skin.¹⁸ To assess whether UV irradiation had any effect on the spontaneous proliferation of DLN cells, mice were irradiated twice a week for 3 weeks and their LN removed 42 h after the final irradiation. No difference was seen in the spontaneous proliferation of cells from UV-irradiated mice compared with unirradiated controls (Fig. 4). This experiment was repeated 3 times with similar results. In one experiment, proliferation of LN cells stimulated with the mitogen concanavalin

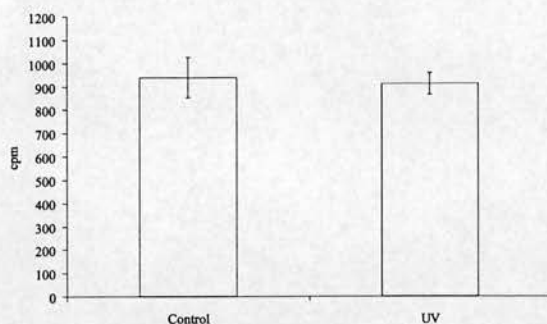


Fig. 4 Effect of 3 weeks UVB exposure on the spontaneous proliferation of DLN cells. Mice (8 per group) were irradiated twice a week for 3 weeks and LN draining the irradiated sites removed 42 h after the final exposure. Control mice were not irradiated. The level of proliferation was assessed using ^3H -thymidine incorporation. Error bars show the SEM.

A ($2.5 \mu\text{g ml}^{-1}$ for 42 h)¹⁹ was measured; the stimulation index (proliferation in response to concanavalin A divided by proliferation without the mitogen) was 37 for both control and irradiated mice.

Exposure to UVB for 3 weeks suppresses the CH response

Suppression of the CH response following multiple UVB exposures has been demonstrated previously.¹³ To assess the effect of the UV exposure protocol used in the present study on the ability of mice to mount an immune response, the CH response to oxazalone was measured. As shown in Fig. 5, mice irradiated with multiple UVB doses showed a significantly suppressed CH response compared with unirradiated controls.

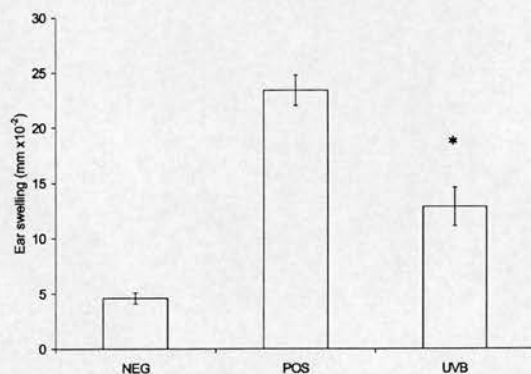


Fig. 5 Effect of 3 weeks UVB exposure on CH response to oxazalone. Irradiated (UVB) or unirradiated (POS) mice (4–6 per group) were sensitised with oxazalone on the dorsal skin 3 days after the final UVB exposure, and challenged 8 days later on the ears. Unirradiated negative control mice (NEG) were challenged but not sensitised. Error bars show the SEM. The asterisk indicates a significant ($p < 0.05$) difference between the irradiated and unirradiated groups.

Discussion

The results of this study show that, following a 3 week UVB radiation protocol, the number of epidermal LC decreased and this decrease was accompanied by a decrease in DC numbers in the DLN, in contrast to the increase that occurred following a single UV exposure.

The effect of chronic UVB exposure on epidermal LC numbers has been reported previously.¹³ The 3 week UV protocol used in the current study also led to local suppression of the contact hypersensitivity response to oxazalone. A correlation between UV-induced LC depletion and down-regulation of

contact hypersensitivity responses has been demonstrated previously,¹ suggesting a role for LC in UV-induced immune suppression. The decrease in epidermal LC numbers following UV irradiation could be due to apoptosis of these cells, resulting from UV-induced DNA damage. *In vitro* irradiation of human LC with low dose UVB resulted in increased apoptotic cell death.⁸ Following *ex vivo* UVB exposure of human skin biopsies, the number of LC in the epidermis decreased compared to unirradiated controls. This decrease could not be attributed to migration however, as the number of LC found in the culture medium decreased following UV irradiation of the skin.²⁰ On the other hand, a number of studies have provided evidence that the decrease in LC numbers following UV exposure is due to the migration of LC from the skin, rather than through the induction of apoptosis. Experiments involving cannulation of draining afferent lymph vessels have demonstrated an increase in lymph flow and cell output after exposure of human volunteers to UVR,⁶ and an increase in the number of LC in afferent lymph draining the skin of UV-irradiated sheep.²¹ In another human study, suction blisters were raised in skin and the number of LC detected in the blister fluid increased following UVB exposure.⁵ Of these, 20–30% showed evidence of UV-induced DNA damage, indicating that they were derived from the UV-exposed epidermis; additionally, very few apoptotic LC were observed in the epidermis and dermis of the irradiated skin. Following a single UV irradiation, mouse cells staining positive for cyclobutane pyrimidine dimers (a specific marker for the commonest form of UV-induced DNA damage) were present in DLN; at least some of these cells had characteristic DC markers, suggesting that they were LC that had migrated from the irradiated skin.²²

In the current study, as reported previously by Moodycliffe *et al.*,⁷ the number of DC in the DLN increased in mice that had received a single dose of UVR, confirming that exposure to UV causes migration of DC. By contrast, following a chronic 3 week UV protocol, the number of DC in DLN decreased compared to unirradiated mice. In the study by Moodycliffe *et al.*,⁷ the increase in DC numbers in the DLN of irradiated mice reached a maximum at 42 h after the exposure, and then decreased rapidly to the levels found in unirradiated mice by around 60 h. This decrease may be due to the re-circulation of the DC; alternatively, the DC that have migrated from the UV-exposed skin may apoptose upon reaching the LN. *In vitro* studies have demonstrated that UVB-irradiated LC only show increased apoptosis following at least 2 days in culture,^{8,9} leading to the suggestion that LC migrating from the skin apoptose once they have reached the DLN. Similarly, following skin painting with an irritant, DC accumulate in the DLN, but disappear rapidly after 2 days,²³ an effect attributed to the death of the DC, since no DC have been observed leaving LN *via* the efferent lymphatics.²⁴ This is in contrast to DC migrating from the skin in unsensitised mice, which demonstrate a turnover of around 30 days.²³ The death of DC in sensitised mice could result from interaction with antigen-specific T cells, as splenic DC showed increased apoptosis when cultured *in vitro* with antigen-specific T cells.²⁵ No increase in the spontaneous proliferation of cells from the DLN of irradiated mice was seen in the present study, indicating that the DC did not stimulate antigen-specific T cells upon arrival; this is to be expected, since the mice were not sensitised. No difference in the level of cell death in DC isolated from unirradiated and irradiated mice was indicated using the marker annexin V; however, a high level of staining was seen in both groups, possibly as a consequence of the *in vitro* manipulation of the cells. It may be that the decrease in both epidermal LC and LN DC numbers in irradiated mice resulted from cell death that occurred earlier in the UVB protocol; alternatively some of the migrating LC could have died in the draining lymphatics.

Despite the decrease in DC numbers in the DLN of irradiated mice, no difference in expression of the cell surface mole-

cules Ia and CD86 was found on DC from DLN of UV-exposed mice, compared to DC from unirradiated mice. Therefore, the DC that remain in the DLN following multiple UV exposures express normal levels of at least two of the molecules involved in antigen presentation to, and stimulation of, T cells. However this finding does not rule out the possibility that UVB may affect the expression of other DC surface molecules such as CD80, CD40 and CD11c, which were not assessed in the present study.

Although we have shown that, following exposure of mice to UV over a 3 week period, the number of LC in the epidermis decreased, there was no evidence that the LC had migrated to the DLN, since the number of DC in the DLN of chronically UV-irradiated mice was significantly decreased compared to unirradiated mice. The ultimate fate of these cells remains to be determined. As seen previously, multiple exposures to UV suppress the contact hypersensitivity response¹³ and result in enhanced outgrowth of implanted tumours in UV-irradiated mice.²⁶ The decrease in both epidermal LC and DC in the DLN suggests that there is a lack of available antigen-presenting cells in chronically UV-irradiated mice, which could either lead to less effective initiation of an immune response, or make antigen presentation by other cells, such as macrophages, more important in these mice.

Acknowledgement

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References

- 1 G. B. Toews, P. R. Bergstresser and J. W. Streilein, Epidermal Langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB, *J. Immunol.*, 1980, **124**, 445–453.
- 2 T. Yoshikawa, V. Rae, W. Bruinsslot, J. W. Vandenberg, J. R. Taylor and J. W. Streilein, Susceptibility to effects of UVB radiation on induction of contact hypersensitivity as a risk factor for skin cancer in humans, *J. Invest. Dermatol.*, 1990, **95**, 530–536.
- 3 M. L. Kripke, C. G. Munn, A. Jeevan, J. M. Tang and C. Bucana, Evidence that cutaneous antigen-presenting cells migrate to regional lymph nodes during contact sensitization, *J. Immunol.*, 1990, **145**, 2833–2838.
- 4 W. Aberer, G. Schuler, G. Stingl, H. Honigsmann and K. Wolff, Ultraviolet-light depletes surface markers of Langerhans cells, *J. Invest. Dermatol.*, 1981, **76**, 202–210.
- 5 W. Kolgen, H. Both, H. van Weelden, K. L. H. Guikers, C. A. F. M. Bruijnzeel-Koomen, E. F. Knol, W. A. van Vloten and F. R. de Gruijl, Epidermal Langerhans cell depletion after artificial ultraviolet B irradiation of human skin in vivo: apoptosis versus migration, *J. Invest. Dermatol.*, 2002, **118**, 812–817.
- 6 N. Yawalkar, M. C. Aebischer, R. Hunger, C. U. Brand and L. R. Braathen, Effects of UV irradiation with one minimal erythema dose on human afferent skin lymph in vivo, *Exp. Dermatol.*, 1998, **7**, 362–368.
- 7 A. M. Moodycliffe, I. Kimber and M. Norval, The effect of ultraviolet B radiation, and urocanic acid isomers on dendritic cell migration, *Immunology*, 1992, **77**, 394–399.
- 8 F. M. Rattis, M. Concha, C. Dalbiez-Gauthier, P. Courtellemont and J. Peguet-Navarro, Effects of ultraviolet B radiation on human Langerhans cells: functional alteration of CD86 upregulation and induction of apoptotic cell death, *J. Invest. Dermatol.*, 1998, **111**, 373–379.
- 9 A. Tang and M. C. Udey, Effects of ultraviolet-radiation on murine epidermal Langerhans cells - doses of ultraviolet-radiation that modulate ICAM-1 (CD54) expression and inhibit Langerhans cell-function cause delayed cytotoxicity in vitro, *J. Invest. Dermatol.*, 1992, **99**, 83–89.
- 10 K. D. Cooper, N. Duraiswamy, C. Hammerberg, E. Allen, C. Kimbroughgreen, W. Dillon and D. Thomas, Neutrophils, differentiated macrophages, and monocyte/macrophage antigen presenting cells infiltrate murine epidermis after UV injury, *J. Invest. Dermatol.*, 1993, **101**, 155–163.
- 11 C. Hammerberg, N. Duraiswamy and K. D. Cooper, Active induction of unresponsiveness (tolerance) to DNFB by in vivo ultraviolet-exposed epidermal cells is dependent upon infiltrating Class II MHC⁺ CD11b (bright) monocytic/macrophage cells, *J. Immunol.*, 1994, **153**, 4915–4924.
- 12 M. S. Duthie, I. Kimber, R. J. Dearman and M. Norval, Differential effects of UVA1 and UVB radiation on Langerhans cell migration in mice, *J. Photochem. Photobiol., B*, 2000, **57**, 123–131.
- 13 A. A. El-Ghorr, M. Norval, M. B. Lappin and J. C. Crosby, The effect of chronic low-dose UVB radiation on Langerhans cells, sunburn cells, urocanic acid isomers, contact hypersensitivity and serum immunoglobulins in mice, *Photochem. Photobiol.*, 1995, **62**, 326–332.
- 14 M. B. Chaker, M. D. Tharp and P. R. Bergstresser, Rodent epidermal Langerhans cells demonstrate greater histochemical specificity for ADP than for ATP and AMP, *J. Invest. Dermatol.*, 1984, **82**, 496–500.
- 15 S. E. Macatonia, A. J. Edwards and S. C. Knight, Dendritic cells and the initiation of contact sensitivity to fluorescein isothiocyanate, *Immunology*, 1986, **59**, 509–514.
- 16 A. M. Moodycliffe, I. Kimber and M. Norval, Role of tumour necrosis factor- α in ultraviolet B light-induced dendritic cell migration and suppression of contact hypersensitivity, *Immunology*, 1994, **81**, 79–84.
- 17 M. B. Lappin, I. Kimber, R. J. Dearman and M. Norval, Exposure of UVB sensitive mice to immunosuppressive doses of UVB in vivo fails to affect the accessory function or the phenotype of the draining lymph node dendritic cells, *Exp. Dermatol.*, 1996, **5**, 286–294.
- 18 I. Kimber, J. A. Mitchell and A. C. Griffin, Development of a murine local lymph node assay for the determination of sensitizing potential, *Food Chem. Toxicol.*, 1986, **24**, 585–586.
- 19 A. A. El-Ghorr and M. Norval, The role of interleukin-4 in ultraviolet B light-induced immunosuppression, *Immunology*, 1997, **92**, 26–32.
- 20 I. B. Kremer, R. M. R. Sylva-Steenland, J. D. Bos and M. B. M. Teunissen, Despite the presence of UVB-induced DNA damage, HLA-DR⁺ cells from ex vivo UVB-exposed human skin are able to migrate and show no impaired allostimulatory capacity, *J. Invest. Dermatol.*, 1997, **109**, 626–631.
- 21 G. W. Dandie, G. J. Clydesdale, F. J. Radcliff and H. K. Muller, Migration of Langerhans cells and $\gamma\delta^+$ dendritic cells from UV-B-irradiated sheep skin, *Cell. Immunol. Biol.*, 2001, **79**, 41–48.
- 22 Y. Sontag, C. L. H. Guikers, A. A. Vink, F. R. de Gruijl, H. Van Loveren, J. Garssen, L. Roza, M. L. Kripke, J. C. van der Leun and W. A. van Vloten, Cells with UV-specific DNA damage are present in murine lymph nodes after in vivo UV irradiation, *J. Invest. Dermatol.*, 1995, **104**, 734–738.
- 23 C. Ruedl, P. Koebel, M. Bachmann, M. Hess and K. Karjalainen, Anatomical origin of dendritic cells determines their life span in peripheral lymph nodes, *J. Immunol.*, 2000, **165**, 4910–4916.
- 24 J. B. Smith, G. H. McIntosh and B. Morris, The traffic of cells through tissues: a study of peripheral lymph in sheep, *J. Anat.*, 1970, **107**, 87–100.
- 25 H. Matsue, D. Edelbaum, A. C. Hartmann, A. Morita, P. R. Bergstresser, H. Yagita, K. Okumura and A. Takashima, Dendritic cells undergo rapid apoptosis in vitro during antigen-specific interaction with CD4⁺ T cells, *J. Immunol.*, 1999, **162**, 5287–5298.
- 26 M. L. Kripke and M. S. Fisher, Immunologic parameters of ultraviolet carcinogenesis, *J. Natl. Cancer Inst.*, 1976, **57**, 211–215.